A DISSERTATION ON

PHYTOCHEMICAL SCREENING, ANTIOXIDANT AND ACETYLCHOLINE ESTERASE (ACHE) INHIBITORY POTENTIAL OF SEQUENTIALLY EXTRACTED *MAZUS PUMILUS*

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BY

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This is to certify that **Ms. Isra Khan**, a student of M. Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled "***Phytochemical Screening, Antioxidant and Acetylcholine Esterase (AChe) Inhibitory Potential of Sequentially Extracted Mazus Pumilus***" successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. M. Salman Khan.**

The dissertation was a compulsory part of her M. Sc. degree. I wish her good luck in future endeavours.

Dr. Snober S. Mir
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This is to certify that the study conducted by **Ms. Isra Khan** during the months Feb–May, 2022 reported in the present thesis was under my guidance and supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled "***Phytochemical Screening, Antioxidant and Acetylcholine Esterase (ACh) Inhibitory Potential of Sequentially Extracted Mazus Pumilus***" therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology, Department of Biosciences, Integral University, Lucknow.

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Date: **ISRA KHAN**

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AD	Alzheimer’s Disease
ROI	Reactive Oxygen Intermediates
PD	Parkinson’s Disease
HD	Huntington’s Disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
NA	Nonadradrenaline
AhT	Choline Transporters
ChE	Cholinesterase
FDA	Food and Drug Association
ROS	Reactive Oxygen Species
NADPH	Nicotinamide Adenine Dinucleotid Phosphate
O^{2-}	Superoxide Anion
H_2O_2	Hydrogen Peroxide
OH	Hydroxyl Radical
ROOH	Organic Hydroleroxide
HOCl	Hypochlorous Acid
SOD	Superoxide Dismutase
LDL	Low Density Lipoprotein
LH	Lipid Hydroperoxide

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Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder among elderly people is characterized by loss of memory, progressive deficits in cognitive functions and behavioral abnormalities (Alvi et al., 2019; Iqbal et al., 2021). It is estimated that more than 36 million people are presently suffering from AD and it still continues to be one of the leading causes of death due to neurological diseases in developed countries. Over the past decades, despite several efforts from various researchers across the globe, its pathogenesis still remains unclear. Several factors including accumulation of β-amyloid, hyperphosphorylation of tau protein, oxidative stress and deficit of acetylcholine (ACh) seem to play a major role in the progression of the disease (Alvi et al., 2019; Iqbal et al., 2021). Current clinical therapy is mainly based on cholinergic hypothesis, which suggests that decline of Ach levels leads to memory loss. Hence sustaining or recovering the cholinergic function is supposed to be clinically beneficial.

Oxidative stress is defined as a state in which the level of toxic reactive oxygen intermediates (ROI) overcomes the endogenous antioxidant defenses of the host. Toxic reactive oxygen intermediates (ROI) are produced by phagocytic cells following injury and inflammation of tissues as a mechanism to kill invading microorganism (Alvi et al., 2017a; 2017b; Bulger et al., 2001). When inflammation becomes systemic as in inflammatory response syndrome there is loss of control of ROI production leading to non-discriminant injury of tissues and organs in the host (Bulger et al., 2001). Oxidative stress has been shown to cause secondary damage through delayed cellular death and inflammation (Alvi et al., 2016; 2019; Ahmad et al., 2019; 2020). Therefore, minimizing oxidative stress may prevent cellular death, decrease inflammation, and prevent some morbidity and mortality (Christman et al., 2000; Nabi et al, 2021).

Free radicals are implicated in oxidative stress reactions, which can damage cells and tissues and cause disorders in the immune system resulting in cancer, diabetes, aging, and cardiovascular and neurodegenerative diseases (Nabi et al., 2021; Akhter et al., 2019; Ahmad et al., 2019; Nabi et al., 2020; Alvi et al., 2021; Ahmad et al., 2022). Antioxidants, therefore, play an important role in disease

prevention and health maintenance. To this extent, there is an increasing role of free radical mediated damage in human disease etiology (Akhter et al., 2019; Alvi et al., 2017a; 2017b). Due to several side effects associated with the already available antioxidants and drugs for AD, there is a need for more selective and potent drugs.

Acetylcholinesterase (AChE) catalyzes hydrolysis of the neurotransmitter acetylcholine, which results in the termination of the nerve impulse in cholinergic synapse, and consequently induces several neurological disorders such as Alzheimer disease (AD) and Parkinson disease (Katzung, 2001). AChE inhibitors are the most effective approach to treat the cognitive symptoms of AD and other possible therapeutic applications in the treatment of Parkinson disease, senile dementia, and ataxia, among others (Atta-ur-Rahman et al., 2004). Inhibition of AChE increases the neurotransmitters in the synaptic cleft and results in a positive cognitive effect in AD patients (Kolari, 2010). Acetylcholine acts as principal neurotransmitter in the central and peripheral nervous system. It plays a role in the transformation of information between a neuron and its adjacent cells (synaps) (Campbell et.al, 2002). AChE inhibitors such as tacrine, donepezil, rivastigmine, and galanthamine are the only drugs currently approved for the treatment of AD. However, due to their short half-lives or unfavorable side effects these drugs are known to have limitations for clinical use (Sung et al., 2002).

It is worth mentioning that tacrine in 1993 received FDA approval as the first approved drug for Alzheimer's disease and acts as an AChE inhibitor; however, due to its various side effects, such as hepatotoxicity, it was discontinued in 2013 for disease treatment (Sharma K, 2019). Similarly, the other approved medications have several side effects, and due to the economic and safety of natural products, there is a continuous need to search for bioactive potential of the natural products or compounds derived from them. Prasad and Muralidhara found that co-administration of geraniol with curcumin has an inhibitory effect on AChE activity Prasad & Murlidhara 2014). Previous reports suggested that natural plant-based products, such as essential oils have shown neuroprotective effects through the inhibition of AChE (Alvi et al., 2019; Oboh et. al., 2014).

Mazus pumilus Linn., commonly called Japanese mazus, is a species of flowering plant in the Mazaceae family (Chi et al., 2018). Mazus is a low–growing perennial plant consisting of 30 species. Mazus pumilus, an annual herb belongs to family Mazaceae, also called Asian mazus or Japanese Mazus. It is generally found in damp habitats in lowland or mountain regions of China, Japan, South East Asia, Australia, New Zealand and Punjab region of Pakistan and India. In Chinese medicine, it is known as “Tong quan cao”. The herb is of great significance owing to its diverse therapeutic benefits. As an ethnomedicine, the leaves of herb have been used in epileptic seizures (Sharma et., al 2013).

This herb possesses antimicrobial activity against certain bacteria and fungi (Safdar et. al 2015). The anticancer activity by the leaf extract of *M. pumilus* on human cell lines have been reported by Priya et al., (2016). The herb is likewise accounted to relieve constipation, a stimulator to menstrual flow, a vigor tonic and antipyretic agent. The juice of the herb is used as a remedy for typhoid fever. In an ethnobotanical review, the herb was discovered as fodder for the livestock (Ishtiyaq et. al., 2018). The plant extract possesses appreciable antioxidant activity, because of which the herb is also mentioned as cardioprotective (Shahid et al., 2013). Because of the anti-oxidative properties of the herb’s extracts, the present investigation was brought up with a target to assess various extracts of *M. pumilus* herb for the AChE inhibitory activity for the treatment and management of neurological disorders.

Review of Literature

Neurological Disorders

Migraine

Migraine is a common, chronic, incapacitating neurovascular disorder, characterized by attacks of severe headache, autonomic nervous system dysfunction, and in some patients, an aura involving neurologic symptoms (Lance and Goadsby, 1998, Silberstein et.al, 1998). Migraine involves dysfunction of brain-stem pathways that normally modulate sensory input. The key pathways for the pain are the trigeminovascular input from the meningeal vessels, which passes through the trigeminal ganglion and synapses on second- order neurons in the trigeminocervical complex. These neurons, in turn, project through the quintothalamic tract, and after decussating in the brain stem, form synapses with neurons in the thalamus. There is a reflex connection between neurons in the pons in the superior salivatory nucleus, which results in a cranial parasympathetic outflow that is mediated through the pterygopalatine, otic, and carotid ganglia. This trigeminal–autonomic reflex is present in normal person (Burstein et.al, 2000) and is expressed most strongly in patients with trigeminal–autonomic cephalgias, such as cluster headache and paroxysmal hemicrania; it may be active in migraine. Brain imaging studies suggest that important modulation of the trigeminovascular nociceptive input comes from the dorsal raphe nucleus, locus ceruleus, and nucleus raphe magnus.

Stroke

Stroke is caused by blockage of a cerebral artery, leading to focal ischaemia, loss of neurons and glial cells, and motor, sensory or cognitive impairments. No effective treatment to promote recovery exists, so a therapy that produced even minor improvement would be valuable. Transplanted cells from different sources, such as fetal brain, neuroepithelial or teratocarcinoma cell lines, bone marrow and umbilical cord, have yielded some improvement in animals and, in one clinical trial, in humans affected with stroke. In most cases, the grafts have acted by providing trophic factors that enhance cell survival and function (Lindvall et.al, 2004).

Parkinson's disease

The pathological hallmark of Parkinson's disease (PD) is a gradual loss of nigrostriatal dopamine-containing neurons, but degeneration also occurs in systems of non-dopaminergic neurons. The main symptoms are rigidity, poverty of movement (bradykinesia), tremor and postural instability. Current therapies centre on the oral administration of l-dopa and dopamine receptor agonists, and on deep-brain stimulation in the subthalamic nucleus. These treatments are effective for some symptoms,but are associated with side effects and do not stop the progression of the disease. To be clinically competitive, a stem-cell-based therapy must lead to long-lasting, significant improvement in mobility, ameliorate currently intractable symptoms, or counteract disease progression.

Huntington's disease

Huntington's disease (HD) is a fatal, intractable disorder that is characterized by chorea (excessive spontaneous movements) and progressive dementia. It is caused by the death of projection neurons in the striatum. Stem-cell therapy aims to restore or preserve brain function by replacing and protecting striatal neurons — a strategy that might be insufficient because patients also suffer progressive neocortical degeneration.

Amyotrophic lateral sclerosis

In amyotrophic lateral sclerosis (ALS), dysfunction and degeneration of motor neurons occur not only in the spinal cord (lower motor neurons) but also in the cerebral cortex and brainstem (upper motor neurons). Muscle weakness progresses rapidly and death occurs within a few years. There is no effective treatment.

Alzheimer's Disease

In Alzheimer's disease (AD), there is a loss of explicit (more than implicit) memory and hypoactivity of cholinergic projections to the hippocampus and cortex (Perry et al 1999).

Alzheimer's disease is a progressive neurogenerative disease that leads to symptoms of dimentia. First names "Alzheimer`s disease" by Emil Kraepelin (Möller et. Al 1998) a German psychiatrist who worked with Dr. Alois Alzheimer.

Approximately 46.8 million people over the age of 60 years have been diagnosed with AD worldwide (Prince et al., 2016). Though occurrence of the early onset of dementia is <1% per 4,000 individuals, the projected figure is estimated to be 131.5 million in 2050 (Prince et al., 2016).

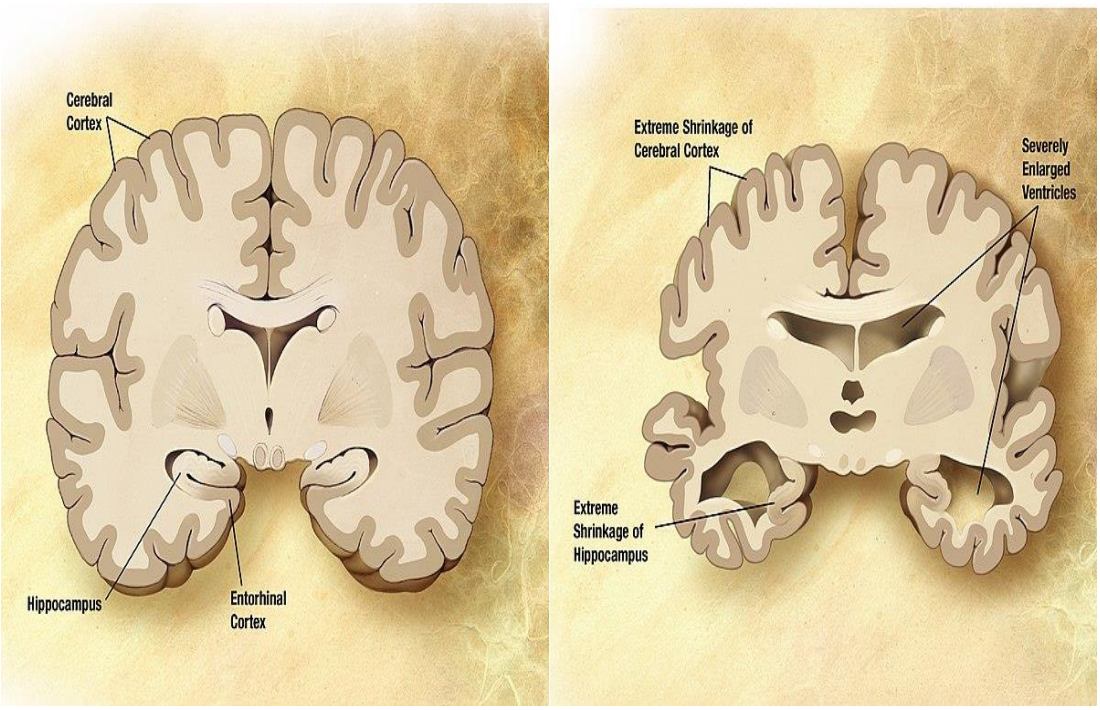


Figure 1: Normal brain on left contrasted with structural changes shown in brain on right of person with Alzheimer's disease, the most common neurodegenerative disease.

The pathophysiology of this disease is quite complex and not entirely understood. Few hypotheses such as cholinergic hypothesis, amyloid hypothesis and Tau hypothesis that try to explain the cause of Alzheimer's disease.

1. Cholinergic hypothesis which states that a possible cause of Alzheimer's is the loss of cholinergic neurons and ensuing deficiency of acetylcholine – a neurotransmitter involved in memory and learning.

2. Amyloid hypothesis which states that Alzheimer's can be caused by accumulation of abnormally folded beta-amyloid proteins. Beta amyloid is a metabolic waste product present in the fluid between brain cells. In Alzheimer's disease beta – amyloid clumps together to form amyloid plaques which are thought to induce neuroimflammation and disrupt communication between neurons.
3. Tau hypothesis which proposes that Alzheimer's may result from abnormal aggregation of Tau proteins which leads to the formation of tangles within nerves cells in the brain. In healthy brain the Tau protein help to lengthen and support microtubule structure. Microtubules play crucial role in transport of nutrients and information molecules throughout the neuron. So when Tau dissociates, the microtubule assembly becomes compromised thereby disrupting the neurons transport system leading to malfunctions in biochemical communication between neurons.

Cholinergic Hypothesis

A classical theory regarding the biological mechanism of Alzheimer's is based on the cholinergic hypothesis (Jantzi, 2010). It is the oldest, most frequently targeted pathway for the treatment of Alzheimer's disease. The brain relays information to other parts of the body through a system of nerve cells, known as neurons. These neurons are typically composed of three parts: the dendrite, the axon and the terminal. The dendrite interacts with the terminal of the preceding neuron at the synapse. When a part of the body needs to be activated, a signal is sent down the system of nerve cells. The pre-synaptic neuron relays the signal to the post-synaptic neuron chemically by releasing neuro-transmitters (molecules that bind to receptor sites on the post-synaptic cell) that tell the post-synaptic neuron to continue sending the signal. Neuro-transmitters are then broken down by enzymes to ensure that the neuron is not over-stimulated (which could lead to cell damage). The signal is transmitted in this fashion until it reaches the part of the body that requires stimulation. A number of neuro-transmitters have distinct roles in different regions of the brain. In normal brain signaling, acetylcholine (Ach) is a neuro-transmitter related to preserving and accessing memory, as well as function.

Ach is broken down by cholinesterase enzymes (ChE): acetylcholinesterase and butylcholinesterase, so that post-synaptic receptors are not over-stimulated and so that Ach does not accumulate in the synapse.

The cholinergic hypothesis postulates that Alzheimer's is caused by a reduction in an individual's ability to synthesize Ach, leading to gradual neuro-degeneration (Francis et. al, 1999). The observed cognitive deficits in Alzheimer's patients with decreased Ach receptor binding led researchers to hypothesize that increasing the availability of Ach in the brain could assuage the cognitive decline associated with Alzheimer's. Administration of a cholinesterase inhibitor (ChEI) decreases the activity of ChE in the synapse, thus leaving more Ach available for signal propagation. Inhibition of ChE explains many of the adverse effects of the ChEIs, as Ach is also an important neuro-transmitter in the digestive tract, the cardiovascular system, and the neuro-muscular junction. As a result, ChEIs may cause nausea, diarrhea, bradycardia, and muscle cramps (APA Practice Guidelines, 2007).

Alzheimer's disease and 'taupathies'

AD involves two major kinds of protein aggregates.

- Extracellular aggregates known as neuritic plaques have as their major constituent the Aβ peptide, which is derived from proteolytic processing of the amyloid precursor protein (APP). The Aβ-containing aggregates have β-sheet structure and Congo red and thioflavin-T reactivity characteristic of amyloid (Serpell and Smith, 2000).
- There are also intracellular aggregates of the microtubule-associated protein tau, called neurofibrillary tangles. The pathogenesis of AD has been greatly clarified by the identification of genetic mutations responsible for rare familial forms of the disease. These mutations are in APP itself and also in the presenilins, which are involved with the cleavage of APP (Esler and Wolfe,2001, Citron, 2002).

Protein Aggregation

Neurodegenerative disease proteins often appear to be natively unfolded. There may be several kinds of aggregates, including disordered or 'amorphous' aggregates, but amyloid fibrils are most characteristic. Covalent modifications of proteins may facilitate aggregation. Sporadic neurodegenerative diseases are generally associated with aging, which is accompanied by oxidative modifications of proteins. Oxidative modification of α-synuclein via dopamine adducts may facilitate aggregation (Conway et.al, 2001). Aging may also decrease the ability of the cell to clear misfolded proteins.

Another important covalent promoter of aggregation is phosphorylation. α-Synuclein purified from Lewy bodies is extensively phosphorylated on Ser129 (refs. 64–67), and experiments in cell culture suggest that Ser129 phosphorylation of α-synuclein strongly modulates interactions between α-synuclein and synphilin-1, and formation of inclusions. Thus, phosphorylation at Ser129 may have a role in the formation of Lewy bodies in PD.

Phosphorylation

Phosphorylation also is involved in aggregation of ataxin-1, the SCA1 gene product. Elimination of a phosphorylation site in ataxin-1 markedly reduced the extent of the behavioral phenotype, inclusion formation and degeneration of Purkinje neurons in the cerebellum in fly and mouse models of SCA-1 (Emamian et.al, 2003). Phosphorylation is also implicated in AD, as a major portion of the neurofibrillary tangles consists of hyperphosphorylated tau protein.

Other covalent protein modifications may also be involved. The role of ubiquitin is described in more detail later, but a ubiquitin-like modifier termed SUMO has recently been shown to be attached at lysines in the N terminus of huntingtin very near the polyglutamine stretch. Modulations by SUMO decreased aggregation, increased nuclear localization and increased neurodegeneration in a fly model of HD (Steffan et.al, 2004).

Protein cleavage

Proteolytic cleavage may have a role in several of the neurodegenerative diseases, including AD. Aβ is generated by the sequential action of β-secretase and γ-secretase (Esler and Wolfe, 2001, Citron, 2002). By contrast, APP can be cleaved normally into a non-amyloidogenic peptide by the combination of α-secretase and γ-secretase. When APP is intact, it has very little tendency to aggregate, but the small cleavage product Aβ has a strong tendency to aggregate. The cleavage site at which γ-secretase acts can vary by several amino acids, and Aβ (Ma and Lindquist, 2002) is less toxic, and also aggregates less, than Aβ (Sunde and Blake, 1998).

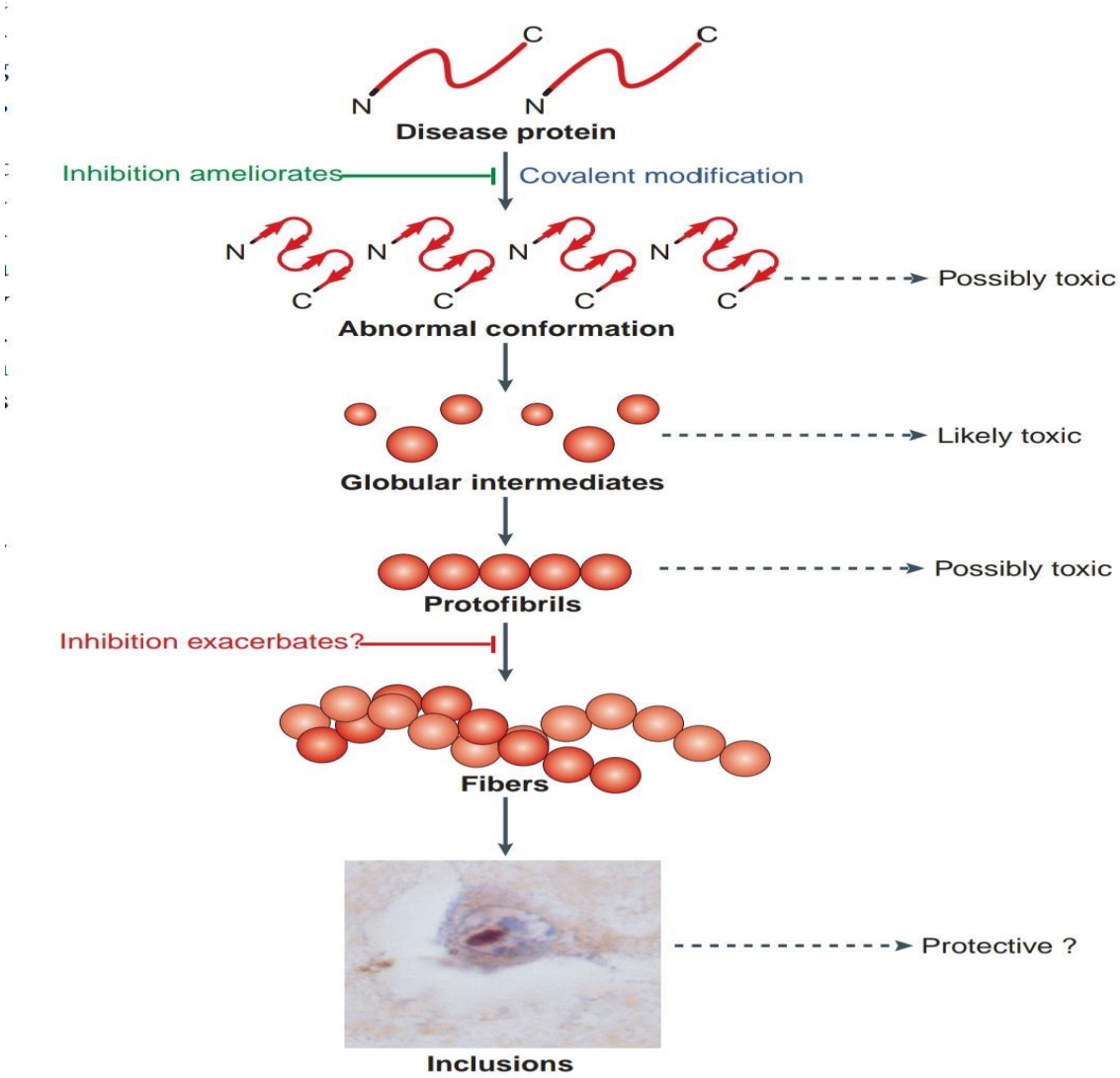


Figure 2: Pathway of protein aggregation.

An initiating event in aggregation may be covalent modification of the disease protein, for example by cleavage or phosphorylation, facilitating conversion of the protein to an abnormal conformation. Oligomeric (globular) intermediates may form, and then protofibrillar structures are assembled. Amyloid fibers can then form, possibly through association of protofibrillar intermediates, resulting in aggregates or inclusions visible in the light microscope. The intermediate species are hypothesized to be more toxic than either the precursor protein orthe aggresomes and inclusions. Inhibition early in the pathway would be beneficial to the cell, because it may prevent the formation of potentially toxic oligomeric or other intermediates. (In a model with linear addition and no oligomeric intermediates, the process of polymerization itself would be presumed to be toxic.) By contrast, inhibition at later stages could be detrimental, because it may result in accumulation of toxic intermediates. If inhibitors could be developed that would act at the intermediate steps, they could help identify which intermediate is the toxic species.

Genetic Risk Factors in Alzheimer's

AD can be classified by the age of onset of the first symptoms. Early-onset AD affects individuals under 65 years of age, accounting for about 4–6% of cases of AD, while the late form AD affects individuals aged 65 years or older. Besides the age of onset of symptoms, the early and late forms of AD differ in other clinical, neuropsychological, neuropathological and neuroimaging variables (Mendez, 2017). Early AD usually occurs due to mutations in genes APP, PSEN1 and PSEN2 (genes of amyloid precursor protein, presenilin 1 and presenilin 2, respectively), whereas late-form AD is mainly associated with a polymorphism in APOE gene (apolipoprotein E gene), especially the presence of ε4 allele (Giri et.al, 2016, Cacace et.al, 2016).

Neurotransmitters

Neurotransmitters are the biochemical compounds, which conducts the signals in the nervous system. The major neurotransmitters in the ANS are acetylcholine and nonadradrenaline (NA) (Clark 2012, Goodman et al 2011, Lodish 2008, Rang 2007).

Acetylcholine

Acetylcholine is the major neurotransmitter in the nervous system, which is an ester of acetic acid and choline. Depending on the type of receptor it interacts, acetylcholine produces either excitatory or inhibitory responses. In the nerve cell cytoplasm the acetyl coenzyme A, which produced in krebs cycle and fatty acid oxidation, combines with choline in presence of acetylcholine transferase to form acetylcholine. This may be targeted in neurological disorders and cardiovascular disorders, overactive bladder, Sjögren's syndrome (Clark 2012, Goodman et al 2011, Rang 2007).

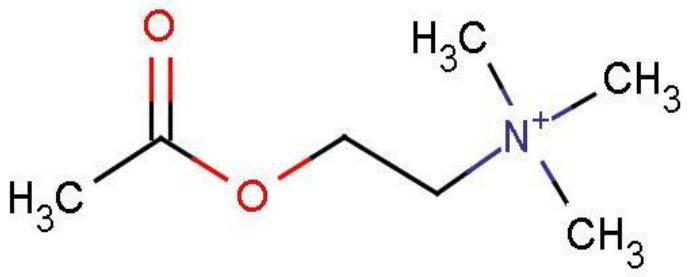


Figure 3: Acetylcholine

Acetylcholine esterase

Acetylcholinesterase (AChE) is the major enzyme that hydrolyzes acetylcholine, a key neurotransmitter for synaptic transmission, into acetic acid and choline (McHardy et.al, 2017) It contains catalytic globular subunits, which are linked to glycolipids or collagen like proteins. To the cell membrane/basement membrane they are chain like a bunch of balloons. It exerts non- catalytic functions in the brain, which are not well studied. Much physiology of acetylcholinesterase enzyme is not so far elucidated very well. Acetylcholinesterase increases the amyloid protein fibrils formation which are toxic and linked to Alzheimer's disease pathology of. Understanding the acetylcholinesterase enzyme physiology is required to find novels therapies to treat neurodegenerative disorders and organophosphate poisonings (Clark 2012, Goodman et.al, 2011, Rang 2007).

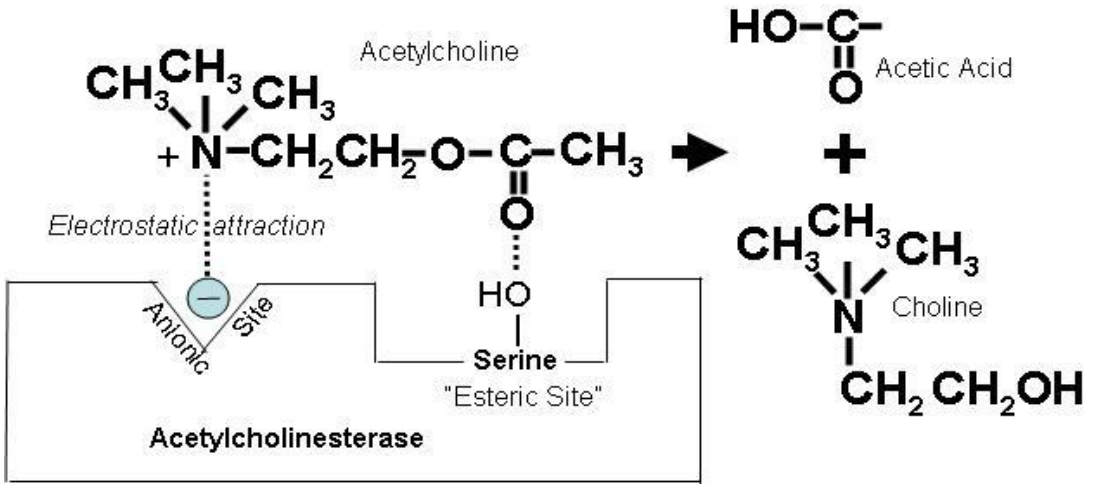


Figure 4: Acetylcholinesterase and breakdown of Acetylcholine. Acetylcholine when binds with AChE the nitrile group of acetylcholine binds at anionic site where as C=O binds the serine hydroxyl group which is esteric site. Then acetylcholine breaks down into choline, which is reuptaken by choline transporters (ChT) into neurone for acetylcholine synthesis.

Organs Involved

Acetylcholinesterase is found in many types of conducting tissue: nerve and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and noncholinergic fibers. The activity of AChE is higher in motor neurons than in sensory neurons (Massoulie et al 1993, Chacho et.al, 1960, Koelle et.al, 1954).

AChE is also found in the red blood cell membranes, where it constitutes the Yt blood group antigen. The enzyme exists in multiple molecular forms, which possess similar catalytic properties, but differ in their oligomeric assembly and mode of attachment to the cell surface. In the mammalian brain the majority of AChE occurs as a tetrameric, G4 form with much smaller amounts of a monomeric G1 (4S) form (Wang et.al, 2005).

Structure of AchE

Knowledge of AChE structure is essential for understanding its high catalytic efficacy and the molecular basis for the recognition of Ach by other Ach-binding protein (Ach receptors), as well as elucidation of the mechanism of action underlying the pharmacological and toxicological action of these agents for the purpose of rational drug design (Sussman et.al, 1991). In the body acetylcholine occurs mainly as a gpi anchored monomer, dimer and tetrameter to nerve and muscles. Each dimer is connected by disulfide bridges between the catalytic subunits. In human cells the main form of acetylcholine esterase is its tetramer. Initial attack by the serine residue on the carboxyl carbon forming a tetrahedral intermediate which is then cleaved by intermediate attack by water.

The active site of AchE comprises two subsites - the ″anionic″ subsite and ″esteratic″ subsites. The anionic subsite accommodates the positive quaternary anime of acetylcholine. The esteratic subsite, where Ach is hydrolyzed to acetate and choline, contains, similar to the catalytical subsites of other serine hydrolases, the catalytic triad of three amino acids: serine 200, histidine 440 and glutamate 327.

Acetylcholinesterase is a serine hydrolase and to rapidly degrade acetylcholine it's one of the most efficient enzymes in the body with It's catalytic efficiency approaching the rate of substrate diffusion. At the bottom of the "active" site gorge is the catalytic triad of acetylcholinesterase. This contains three crucial residues – serine 200 which is coherently bonded to acetylcholine esterase, histidine 440 which removes a proton from serine and lastly glutamate residue which is contrast to aspartate residues normally found in serine hydrolases but it serves as the same function as it hydrogen bonds to histidine – making it a better base and keeping it in place.

Mechanism of action of AchE action in neurotransmission

Cholinergic neurotransmission can be broken down into six major steps. Ach is synthesized by a single step reaction catalyzed by the biosynthetic enzyme choline

acetyltransferase and the presence of this enzyme is the “marker” that a neuron is cholinergic.

- Choline molecule is transported into the neuron by the energy and sodium dependent transport system. Once inside choline reacts with acetyl coenzyme A to form acetylcholine. The enzyme responsible for catalysing this is choline acetyl transferase.
- In the second step, acetylcholine gets transported into pre synaptic vesicle where it is protected from degradation.
- In the third step the action potential causes the voltage- sensitive calcium channels to open thus allowing calcium to enter the axon. This in turn leads to the fusion of the vesicle with the membrane and release of the acetylcholine from the terminal.
- In the fourth step acetylcholine binds to postsynaptic receptors which leads to cholinergic response. It also binds to presynaptic receptors and inhibit the release of more acetylcholine. This serves as a negetive feedback loop.
- In the fifth step, enzyme called acetylcholine esterase terminates acetylcholine function in the synaptic cleft by breaking it down to acetate and choline.
- Finally in the sixth step the free choline is taken up again by the pre – synaptic neuron and the whole gets repeated (Katzung 2001, Bernard 1994).

Cholinergic Receptors

The cholinergic receptor name itself defines the physiological function regulated mainly by the chemical messenger called acetylcholine. The receptors involved are muscarinic receptors and nicotinic receptors.

Acetylcholine receptors are nicotinic receptors and muscarinic receptor –

Nicotinic receptors

Nicotinic receptors are fast excitatory receptors. They mediate fast synaptic transmission since they are directly coupled to cation channels. They are present at the various sites in the central nervous system, autonomic ganglia and at the

neuromuscular junction. Pharmacology and structure of neuronal and muscle nicotinic receptors are different.

Muscarinic receptors

Muscarinic receptors and nicotinic receptors occur both presynaptically as well as postsynaptically. Presynaptic receptors act as autoreceptors and they regulate the neurotransmitter release (Clark 2012, Goodman et al 2011, Rang 2007).

Cholinesterase inhibitors

ChE inhibitors in AD therapy the prominent role of central cholinergic pathways in learning and memory and the correlation of severe cholinergic deficits with cognitive impairment in AD patients contributed to the development of symptomatic cholinergic therapy. Cholinesterase inhibitors block the action of the enzyme cholinesterase, which is responsible for breaking down acetylcholine. This increases levels of acetylcholine in the synaptic cleft (the space between two nerve endings). Besides the degeneration of subcortical cholinergic projection neurons in AD, post-synaptic mAChRs expressed by cholinergic target cells are preserved in AD cortex and hippocampus (Nitsch et al1998, Perry et al 1990, Perry et al 1986, Smith et al1988).Thus, cholinomimetic compounds were designed to stimulate post-synaptic cholinergic target neurons. Cholinesterase inhibitors (ChEIs), such as tacrine, rivastigmine, donepezil and galanthamine have been used in many countries for the symptomatic treatment of mild to moderate AD (Nordberg 1998).

The ChEIs act by inhibiting the degradation of acetylcholine (Nordberg et al 1998). The clinical efficacy of these drugs is characterized by cognitive, functional and global improvements in AD patients (Nordberg et al 1998, Giacobini 2000). It appears that the anti-cholinesterase treatment reduces the progression rate of the disease (Nordberg 1998, Giacobini 2000).

Pharmacokinetic and pharmacodynamic properties

The reversible inhibitors like tacrine, donepezil and galantamine, inhibit AChE by binding to hydrophobic binding sites126. These inhibitors bind to and leave the

enzyme intact, i.e., they are not transformed by the reaction. For a reversible inhibitor, the drug concentration and enzyme inhibition are mirror images of each other. The duration of action is directly linked to the pharmacokinetic properties of the drug, i.e. to the rate at which it is eliminated, and the drug has to be present in order to cause inhibition. The carbamates (e.g. rivastigmine, physostigmine) and organophosphates (e.g.metrifonate) all inhibit AChE by binding to the esteratic site, the catalytic site, of the enzyme (Nordberg et al 1998). One part of the molecule binds covalently to the enzyme while the other part, the leaving group, is split off. Due to this cleavage of the molecule upon binding to the enzyme, there is no relationship over time between pharmacokinetic and pharmacodynamic properties of such inhibitors. The enzyme can be inhibited for a long period after the parent drug, the inhibitor, has been eliminated (Weinstock et al 1994, Bar-On et al 2002).

Cholinesterase inhibitors are divided into two classes, which includes organophosphorus compounds and carbamates. Organophosphorus compounds seem to be more toxic and have a longer duration of action. Carbamates are less toxic and have a rather short duration of action. The structural and functional differences are depicted in the following figure. When organophosphates (insecticides) act on acetylcholinesterase enzyme they act on active site, which is partially electronegative. During transition, the functional group RL, which is attached to the electropositive phosphorous, will be broken down. The partially electropositive phosphorous and partially electronegative oxygen form the complex, which prevents the acetylcholinesterase to bind with acetylcholine. Thus, organophosphate poisonings block the serine functional site in the enzyme. This is shown in the figure (Klinkenberg et al 2013).

Physostigmine

Physostigmine also known as Eserine is an alkaloid present in physostigma venenosum (calabar bean- fabaceous plant). The structure of physostigmine is 1,2,2,3a,8a,-hexahydro-1,3a,8-trimethyl-pyrrolo[2,3-b]indo-5-ol-methylcarbamate. It is a carbamate inhibitor of acetylcholinesterase (Traub et al 2002). Physostigmine salicylate inhibits the acetylcholinesterase reversibly and increase the acetylcholine

concentration at the synapse and stimulates the cholinoceptors; i.e., both muscarinic and nicotinic (Scheindlin 2010). Physostigmine may directly acts on acetylcholine receptor complexes, as well as interact with the acetylcholine gated cation channels. Physostigmine has short duration of action. Physostigmine is non-ionic, tertiary amine which is a lipid soluble compound. Thus physostigmine easily cross the blood brain barrier and may thus act on central nervous system. This property makes to play a key role in the treatment of alzheimer's disease. Physostigmine is clinically used to treat glaucoma, myasthenia gravis etc. Half-life of physostigmine in rat is 16min, in dog 23min where as in man it is 30min. It is metabolised in liver and the bioavailability is very low<2%.

It has narrow therapeutic window (Mach et al 2004, Meshulam et al 2001, Somani & Dube 1989). As a pseudosubstrate or competitor physostigmine interacts with acetylcholinesterase, thus forms the stable intermediate drug-enzyme complex which prevents endogenous acetylcholine from reacting with the acetylcholinesterase enzyme and thus retain the local concentration of acetylcholine at the synapse (Triggle. et al 1998).

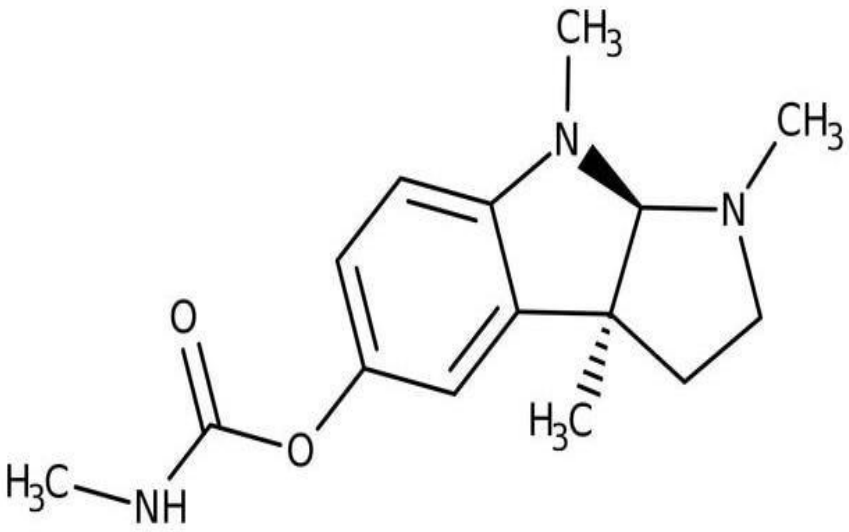


Figure 5: Physostigmine

Donepezil

Donepezil a specifically designed piperidine derivative with reversible acetylcholinesterase inhibitor activity. It has a much higher specificity for acetylcholinesterase inhibition compared with tacrine (Sugimoto et al., 1992) and its CNS selectivity is highlighted by the lack of activity in peripheral tissue such as cardiac tissue or gut smooth muscle (Rogers et al., 1991). The pharmacokinetics are linear and dose proportional, with peak plasma levels after approximately 4 h. Plasma steady state appears to be reached between 14 and 21 days with a long half-life of over 70 h (Bryson et al., 1997). Excretion is slow and occurs via renal and the cytochrome P450 system although it is not impaired in patients with hepatic or renal impairment (Rogers et al., 1997).

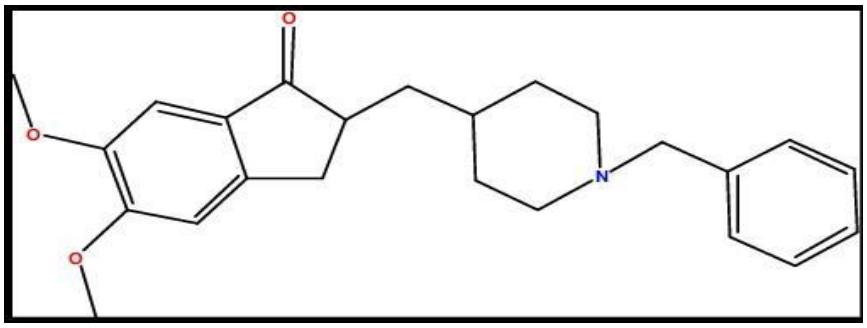
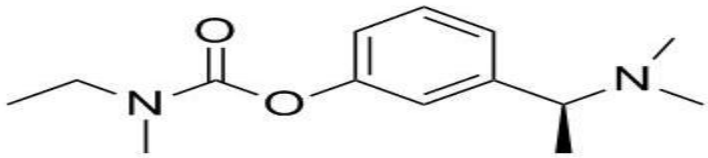


Figure 6: Donepezil

Rivastigmine

Rivastigmine is a pseudo irreversible non – competitive carbamate inhibitor of AChE because it mimics Ach by binding with the enzyme Ache forming a carbamylated complex. This prevents further enzyme-catalysed hydrolysis of Ach for several hours after the drug has been eliminated from the plasma (Anand 1996, Anand and Gharabawi 1996). Although it's half life is approximately 2 hours. The inhibitory property of this agent last for 10 hours because of the slow dissociation of the drug from the enzyme (Anand 1996, Anand and Gharabawi 1996). Food and Drug Association (FDA) approved it's use in mild to moderate Alzheimer disease in April, 2000.



Early and continued treatment of AD with rivastigmine maximizes the observed

Figure 7: Rivastigmine

beneficial effects in the rate of decline of cognitive function, activities of daily living, and severity of dementia with daily doses of 6 to 12 mg. Adverse events are consistent with the cholinergic actions of the drug, and include nausea, vomiting, diarrhea, anorexia, headache, syncope, abdominal pain and dizziness (Birks et al., 2009, Inglis et al., 2002).

Galantamine

Galantamine is an alkaloid extracted from tuberous plant *Leucojum aestivum*. It is a reversible cholinesterase inhibitor that appears to have no effect on butyrylcholinesterase (Kitisripanya et al., 2011, Bartolucciet al., 2001).

In addition, it acts at allosteric nicotinic sites, further enhancing it's cholinergic activity. Galantamine undergoes slow and minor biotransformation with approximately 5% to 6% undergoing demethylation. It is primarily excreted in the urine (Wessler et al., 2008, Pohanka 2011).

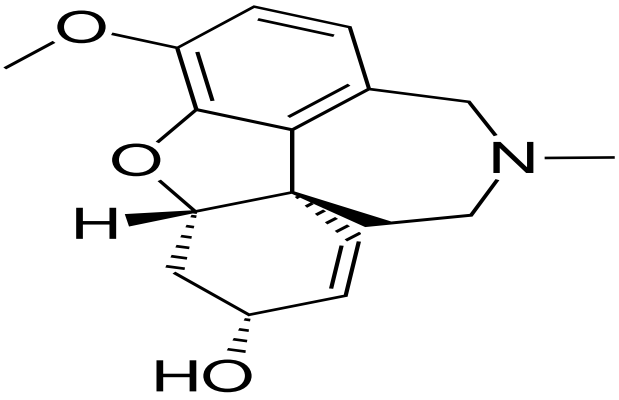


Figure 8: Molecular structure of galantamine

Table 1: Characteristic of some AChE inhibitors

Drug	Type	Half - life (Plasma)	Dose
Physostigmine	**Alkaloid**	**30 min**	**Six to seven times daily**
Tacreine	**Aminoacridine**	**1.4-3.6 h**	**Four times daily**
Donepezil	**Piperidine**	**70 h**	**Once daily**
Rivastigmine	**Carbamate**	**1 h**	**Twice daily**
Metrifonate	**DDVP (active metabolite)**	**2 h**	**Once daily**

Free radical

Free radicals are unstable, short-lived species with an unpaired electron. Free radicals are produced in the body as by product of normal metabolism and as a result of exposure to radiation and some environmental pollutants. Free radicals can produce different effects in different areas of the body because of the difference in chemical structure it can react with anything in its vicinity. Free radicals are highly reactive and in excess it can damage virtually all cellular components. In healthy individuals a delicate balance exists between the levels of free radicals and antioxidants when this balance is disturbed and when it is in favour of reactive of oxygen species it can play an important role in the causation of disease in some pathological conditions such as diabetes mellitus. Body has developed defense to fight against free radicals.

Enzymatic antioxidants and non-enzymatic antioxidants ensure mopping of the free radicals as they are produced (Frie et al., 1988)

Upon collision of a free radical with other molecules they abstract or donate electron in order to gain stability. This leads to the generation of radical form of a molecule with which the free radicals have collided leading to a chain reaction

Important characteristic of reactive oxygen species

- Extreme reactivity
- They have a short life span
- Mostly act where it is produced
- They are involved in the generation of new reactive oxygen species through chain reaction they damages various molecules thus causing the damage to the cells and to the tissues.

Generation of free radicals

Free radicals can be generated in healthy humans.

Electron leakage in Mitochondria

The mitochondria are a principal source of endogenous oxidants (Beckman and Ames, 1998). Mitochondrial respiration utilises oxygen to produce ATP via several reactions including the electron transport system. It appears however, that mitochondria electron transport leaks a small amount of electrons and one-electron reduction of oxygen to form 0_2•- occurs. The spontaneous and enzymatic dismutation of 0_2•- produces H202, thus a significant by-product of the actual sequence of oxidation-reduction reactions may be the generation of 02• and H202 (Beckman and Ames, 1998).

Neutrophils

During illness, phagocytic cells, as a mechanism to kill invading microorganisms, produce ROI. Leakages could result from incomplete phagocytosis of large structures, or disintegration of phagocytes by toxins causing irreparable damage to the cell structure and function (Weiss, 1986). Neutrophil rupture and the resulting leakages have been associated with severe inflammation and multiple injury (Fritz et.al, 1989).

A positive correlation exists between myeloperoxidase, elastase levels and the Acute Physiology and Chronic health Evaluation 3 (APACHE3) (Alonso et.al, 2000). Neutrophils possess antioxidant mechanisms that protect against host injury by the potent products of phagocytosis. These include superoxide dismutase, glutathione peroxidase, catalase, vitamin E and ascorbic acid. They play a significant role in limiting tissue destruction caused by proteases however neutrophil oxidants are known to be the single most important direct mediators of immune injury (Weiss, 1986).

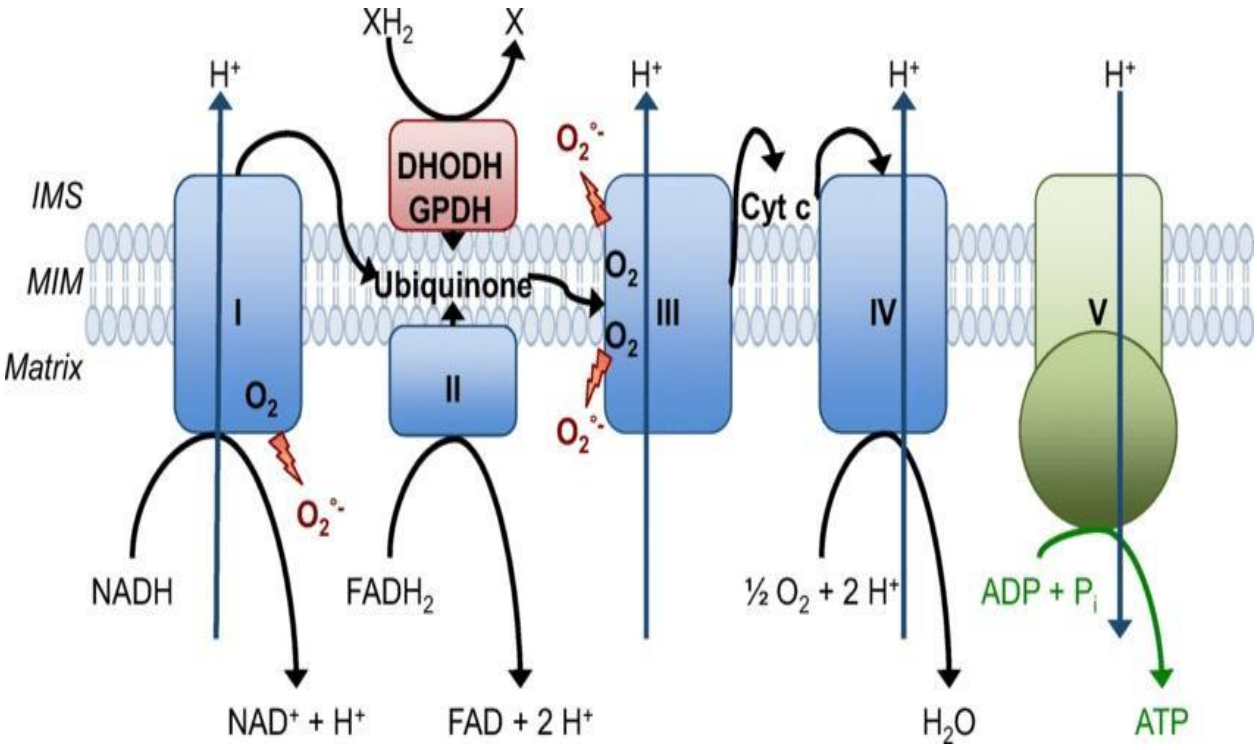


Figure 9: ROS production via electron transport chain

Transition metals

Transition metal ions are important in the production of ROS. The ability of metal ions to donate and accept single electrons is the basis for the formation and propagation of many ROS. Both copper and iron gain or lose electrons during redox reactions, cycling from reduced to oxidised forms and back. Most iron in the body is stored in the oxidised (ferric form).

Fe 2+ (reduced iron) - Fe 3+ (oxidised iron)

Cu+ (reduced copper) - Cu2+ (oxidised copper)

In the presence of transition metal ions H202, which is continuously produced in vivo, easily breaks down to produce the OR radical. This is known as the Fenton reaction, which can also occur with copper and some other metal ions and is usually written as follows (Halliwell et al, 1992).

Exogenous sources

There are numerous exogenous sources of reactive oxygen species. Various compounds such as carbon tetrachloride is capable of generating oxidative stress.

- Ionising radiations damages tissues by producing free radicals.
- Light of certain wavelength cause photolysis of covalent bonds to produce free radicals.
- Cigarette, smoke also contain dangerously high concentrations of free radicals.
- Other exogenous sources of reactive oxygen species include polluted air, water, alcohol, transition metals, heavy metals, drugs as cyclosporine, other industria solvent l cooked items and cooking medium such as repeatedly used oils and fats.

Classification of Reactive Species

RS can be divided into different categories – ROS and reactive nitrogen species (RNS), but also reactive chlorine, bromine and sulphur species. These categories contain both radicals and non-radicals. Superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$) and peroxyl ($RO_2^{\bullet}$) are examples of major ROS radicals. H_2O_2 and singlet O_2 are examples of non-radical ROS. Nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are examples of RNS radicals and peroxynitrite ($ONOO^-$) is an example of a non radical RNS. A radical is a species capable of independent existence that contains one or more unpaired electrons in the atoms orbital. Since the radical strives to become a stable molecule by filling the orbital with an electron it is often a reactive substance.

Superoxide anion O_2^-

Superoxide radical is produced when a single electron is transferred to oxygen. It is an anion as well as a free radical. Superoxide anion can also initiate the cascade of arachidonic acid metabolism, resulting in the formation of more superoxide and in the liberation of Fe2+ from ferritin stores (Yoshida et al., 1995). The enzyme superoxide dismutase (SOD) converts superoxide to hydrogen peroxide and molecular oxygen; however, superoxide can also spontaneously dismutate to form hydrogen peroxide and singlet oxygen, a strong oxidizing agent (Witztum et al., 1994).

Hydrogen peroxide H_2O_2

Hydrogen peroxide is formed by the two-electron reduction of oxygen. Since the reaction generates a non radical species from the radical reactant it termed as dis mutation reaction. This mutation can occur spontaneously or it can be catalyzed by superoxide dismutase. The three-electron reduction product of oxygen is hydroxyl radical which is the most powerful free radical.

Hydroxyl radical OH

Tree-electron reduction state. Formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components. The hydroxyl radical is the most powerful oxidant formed in the biological system with a diffusion ratio of 0.3 nm before it abstracts an electron from another molecule. A single hydroxyl radical and molecular oxygen can react with a poly-unsaturated fatty acid not only altering itís structural and functional integrity but also generating multiple fatty acid peroxyl radical which spontaneously reacts with other lipids, proteins, or nucleic acids thereby propagating a cascade of electron transfer and the consequent oxidation of these substances (Imlay et al., 1988).

Organic hydroleroxide ROOH

Formed by radical reaction with cellular components such as lipids and nucleases.

Hypochlorous acid HOCl

Formed from hydrogen peroxide by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize proteins constituents including thiol groups, amino groups and methionine. Low quantities of reactive oxygen species are formed during normal metabolism. Auto oxidation of thiol, ascorbic acid or glucose are greatly amplified through participation of transition metals and this causes generation of reactive oxygen species.

Oxidative stress

Reactive oxygen species become dangerous to the human body when they produce oxidative stress. Oxidative stress is the enhancement of the body's production of free radicals and other strong oxidants either by increased production of the radical or depletion of body stores of antioxidants. Oxidative stress arises from an imbalance between reactive oxygen species and antioxidants. This imbalance is in favour of excessive generation of free radicals. Increased oxidative stress causes oxidation of biomolecules with consequent loss of their biological functions. Build up of reactive oxygen species whether they are endogenous or exogenous may result in lipid peroxidation, protein oxidation and DNA damage. Free radicals generally react with the first structure they encounter- most frequently the lipid components of cell (Kim et al., 1985).

Damage to nucleic acid by ROS

Nucleic acids – DNA and RNA are highly prone to hydrolysis, non – enzymatic methylation and oxidation. DNA can be damaged by reactive oxygen species by two ways: ROS can modify base or sugar.

- ROS modifies by adding hydroxyl group to the and the hydroxylated bases are different from the normal base.
- Modification of sugar causes the strand breaks.

DNA be modified in many ways. If it is not prepared can ultimately lead to mutation and genomic instability. This could result in the development of variety of cancers including colon, breast and prostate.

Damage to lipid by ROS

Low density lipoprotein are major carriers of cholesterol to the body tissues. In the oxidation of LDL both protein and lipids undergoes oxidative changes that can cause cholesterol accumulation.

Poly unsaturated fatty acids and particular arachidonic and linoleic acid are other targets of lipid peroxidation as it proceed in a self amplifying manner referred to as “chain reaction”.

- Initial reaction of PUFD free radicals generates more free radicals.
- Propagation reaction in which the fatty acid radical reaction in which the fatty acid radical react with oxygen and
- Termination step in which the two reactants they collide with each other to form a non-radical species.

Initial reaction starts with the attack of free radical on one of the methylene hydrogen. The hydrogen between the two double bonds is highly reactive. It takes hydrogen from fatty acid or lipid molecule and gets converted into water and the lipid molecule is converted into its radical form thus initiating the chain reaction. With the interaction of polysaturated fatty acid with free radical, the removal of hydrogen generates the radical form of the lipid which is” carbon centered” and this is termed as the initiation phase.

The peroxidation chain reaction propagates itself once it has started. The process by which lipid radicals (L-) are generated from lipids(LH) is called the chain initiation reaction. Lipid radicals (L-) thus generated react immediately with oxygen, resulting in the formation of LOO-, which attacks another lipid and removes a hydrogen atom from it, resulting in the formation of lipid hydroperoxide (lipid peroxiide; LOOH) and another L . This new L- Also reacts with oxygen and forms LOO-, which attacks another lipid to generate lipid peroxide, so lipid peroxide accumulates as the chain reaction proceeds. Lipid peroxidation disrupts the normal structure and function of the lipid bilayer surrounding both the cell itself and in the membrane of organelles. In

particular, it can alter the membrane permeability, transportation and fluidity (Yoshikawa 1998, Yoshikawa 1997, Yoshikawa 1997).

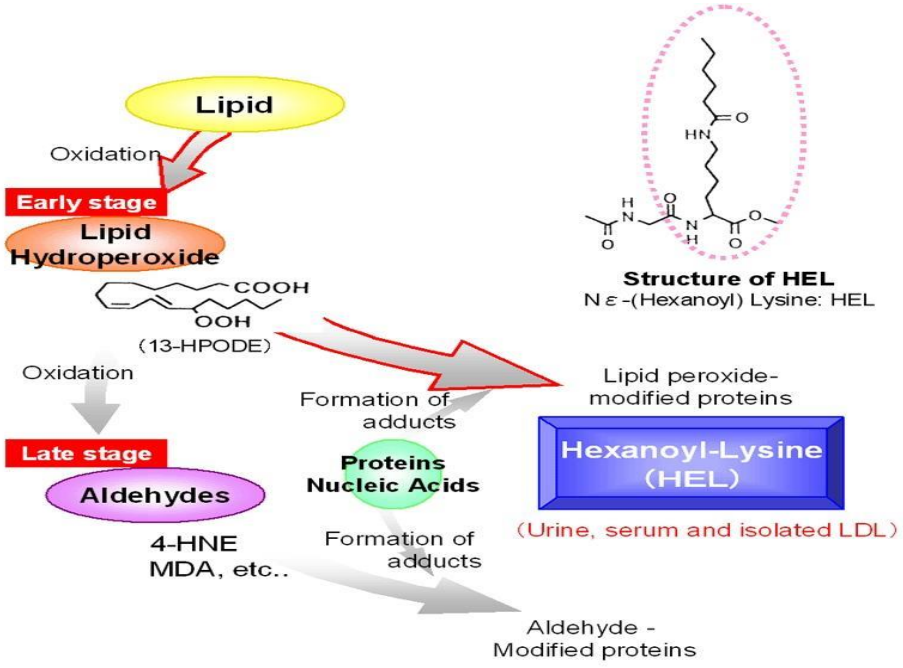


Figure 10: Mechanism of lipid peroxidation: Lipid peroxidation end products as a key of oxidative stress.

Damage to proteins by ROS

Several oxidative damage to proteins has been described. The self-hydral group of cysteine is highly prone to oxidation and can result in the formation of disulfide bridge (Alvi et al., 2021).

Oxidative stress in the nervous system

The nervous system – including the brain, spinal cord, and peripheral nerves – is rich in both unsaturated fatty acids and iron. The high lipid content of nervous tissue, coupled with its high aerobic metabolic activity, makes it particularly susceptible to oxidative damage. In addition, those brain regions that are rich in catecholamines are exceptionally vulnerable to free radical generation. The catecholamine adrenaline, nonadradrenaline, and dopamine can spontaneously break down (auto-oxidise) to free

radicals, or can be metabolised to radicals by the endogenous enzymes such as MAO (monoamine oxidases). One such region of the brain is substantia nigra (SN), where a connection has been established between antioxidant depletion (including GSH) and tissue degeneration (Perry et. al, 2002).

Antioxidants

Antioxidants are substances that neutralize or remove free radicals by donating an electron. The neutralizing effect of antioxidants help protect the body from damage caused due to oxidative stress. An antioxidant is any substance that when present in low concentration as compared to those of an oxidizable substrate significantly delay or prevent the oxidation of that substrate.

Classification of Antioxidants

ENZYMATIC

Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Wuerges et al, 2002, Johnson and 1987). SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Bannisteet al, 2005).

There are three major families of superoxide dismutase, depending on the metal cofactor -

- Cu/Zn (which binds both copper and zinc)
- Fe and Mn types (which bind either iron or manganese), and
- Ni type which binds nickel (Zelko et.al, 2004).

Catalase

Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al, 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted

into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gaetani et.al, 1996).

Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms (Meister and Anderson, 1983). Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals (Brigelius, 1999). Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S-transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes et.al, 2005).

NON ENZYMATIC

Ascorbic acid

Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin (Smirnoff, 2001). Most other animals are able to produce this compound in their bodies and do not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (Meister, 1994). Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide (Padayatty, 2003).

Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life (Meister and Anderson, 1983). It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized

and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants (Meister, 1988). Due to its high concentration and central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants.

Melatonin

Melatonin, also known chemically as N-acetyl-5-methoxytryptamine is a naturally occurring hormone found in animals and in some other living organisms, including algae (Caniato et.al, 2003). Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier (Reiter et.al, 1997). Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Barbas, 2000).

Tocopherols and tocotrienols (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera et, al, 2001). α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber and Atkinson, 2001). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidized α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol, or ubiquinol (Wang and Quinn, 2007).

Uric acid

Uric acid accounts for roughly half the antioxidant ability of plasma. In fact, uric acid may have substituted for ascorbate in human evolution (Jaeschke et al, 2002). Like ascorbate, uric acid can also mediate the production of active oxygen species.

Table 2: Classification of antioxidants based on the basis of their location

Intracellular	Extracellular	Membrane associated
Catalase	Catalase	Alpha -tocopherol
Glutathione	Glutathione	Retinoids
Melatonin	Melatonin	Ubiquinone
Glutathione peroxidase	Glutathione peroxidase	
Ascorbate	Ascorbate	
DNA repair enzymes	Beta carotene	
Thioredoxin	Uric acid	
Lipoic acid	Ceruloplasmin	

Mechanism of Antioxidant Protection

The mechanisms of antioxidant protection can be classified into four categories: compartmentation, detoxification, repair and utilization. Compartmentation is both the spatial separation of potentially harmful but essential substrates (e.g., storage of iron in ferritin) and cell and tissue distribution of antioxidative compounds and serves the prevention of uncontrolled oxidation (Popov et al., 1992). The most significant mechanism from the therapeutic point of view is detoxification because it results in the breakdown of free radicals to non-toxic substances (oxygen, water), thus protecting from many diseases. Detoxification of oxidative molecules i.e., radicals and peroxides, is ensured by enzymatic and non-enzymatic substances. The detoxification enzymes are present intra- and extra-cellularly and protect cells from the destructive side effects of free radical chain reaction (Popov et al., 1992).

Significance of Antioxidants

Antioxidants donate an electron to a free radical and neutralize them without becoming a reactive entity themselves. This causes severe reduction in the reactivity of the free radicals. The unpaired electron in the antioxidant is delocalized and therefore it is not reactive.

Antioxidants protect cells and safeguard their constituent from damage caused by highly reactive free radicals. Every antioxidant behaves differently due to different chemical properties. Some antioxidant can become pro-oxidant such as vitamin C which can grab electron from other molecules create chemical instability and may produce oxidative stress. Although vitamin C is an antioxidant capable of reactant with superoxide and hydroxyl radical mono dehydroascorbate and hydrogen peroxide but it can also be a superoxide radical by reaction with oxygen.

Japanese mazus

Mazus is a low–growing perennial plant consisting of 30 species. Mazus pumilus, an annual herb belongs to family Mazaceae, also alled Asian mazus or Japanese Mazus. It is generally found in damp habitats in lowland or mountain regions of China, Japan, South East Asia, Australia, New Zealand and Punjab region of Pakistan .In Chinese medicine, it is known as “Tong quan cao”. The herb is of great significance owing to its diverse therapeutic benefits. As an ethnomedicine, the leaves of herb have been used in epileptic seizures (Sharma et.al, 2013). The herb possesses antimicrobial activity against certain bacteria and fungi (Safdar et.al, 2015). The anticancer activity by the leaf extract of M. pumilus on human cell lines have been reported (Priya et.al,2016).

The herb is likewise accounted to relieve constipation, a stimulator to menstrual flow, a vigor tonic and antipyretic agent. The juice of the herb is used as a remedy for typhoid fever. In an ethnobotanical review, the herb was discovered as fodder for the livestock (Ishtiaq et.al, 2018). The plant extract possess appreciable antioxidant activity, because of which the herb is also mentioned as cardioprotective (Shahid et.al, 2013). Because of the anti-oxidative properties of the herb’s extracts, the present investigation was brought up with a target to assess methanol extract of M. pumilus herb for the anti-nociceptive, anti-inflammatory and hepatoprotective activities.

Table 3: Scientific classification of J*apanese mazus*

Scientific	***Classification***
Kingdom	*Plantae*
Order	*Lamiales*
Family	*Mazaceae*
Genus	*Mazus*
Species	*M. pumilus*
Binomial name	*Mazus pumilus*

Figure 11: *Japanese Mazus*

Objectives

I. **Collection, identification and preparation of Plant material (*M. pumilus* Linn.).**

II. **Solvent based extraction and phytochemical screening of *M. pumilus* Linn.**

III. **To determine the *in-vitro* antioxidant activity of different fractions of *M. pumilus* Linn. by DPPH radical scavenging assay.**

IV. **To determine the *in-vitro* antioxidant activity of different fractions of *M. pumilus* Linn. by ABTS radical scavenging assay.**

V. ***To delineate the in-vitro* anti-acetylcholinesterase (AChE) inhibitory activity and enzyme kinetic studies of *M. pumilus* Linn extract/s.**

Materials and Methods

Chemicals

Chemicals such as *n*-hexane, ethyl acetate (EtOAc), dichloromethane (DCM), methanol (MeOH) were obtained from Merck, India.1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS was purchased from the Hi Media Laboratories, Mumbai, India. DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), Acetylcholine iodide (AchI), 9-Amino-1,2,3,4-tetrahydroacridine hydrochloride (Tacrine hydrochloride) and acetylcholinesterase (AChE) were purchased from Sigma Aldrich USA. All the other chemicals used in this study were of analytical grade.

Collection, processing, and solvent-based extraction of *M. pumilus* Linn.

M. pumilus Linn. whole plant was collected from the local area around Integral University, Lucknow, India, in the month of February. The plant was botanically identified and authenticated at the Department of Pharmacy, Integral University, Lucknow, India. M. pumilus Linn. whole plant was shed dried and made in coarse powder, avoiding sun light due to the signature modification of the biochemicals. The dried powder (25 g) of the plants was extracted using nonpolar, partially polar, and polar solvents successively with the required amount of each of n-hex, DCM, EtOAc, MeOH, and water in Soxhlet apparatus until it turned colorless. The extract was filtered and dried at room temperature.

Percentage yield of the sequentially extracted plant in different solvent systems was calculated by using the formula:

$$\%\, Yield \;=\; \frac{Weight\ of\ crude\ extract\ (g)}{Weight\ of\ raw\ material\ (g)} \times 100$$

Phytochemical screening

Phytochemical screening consists of tests for phenols, alkaloids, tannins, flavonoids, saponins and triterpenoids, steroids, and cardiac glycosides (Hashim et al., 2013).

Test for phenols

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for color development.

Test for tannins

10 mg sample was boiled in 50 mL of distilled water and then filtered. A quantity (5 mL) of test solution was added into a test tube following some drop of $FeCl_3$. Formation of brownish green or blue, black coloration indicates presence of tannins.

Test for flavonoids

10 mg sample was mixed with 10 mL of distilled water. The mixture was heated for 5 minutes and filtered. The filtrate was mixed with Mg powder, 1 mL of strong HCl and 1 mL of amyl alcohol. Formation of color in the amyl alcohol layer indicates flavonoids.

Test for saponins

10 mg sample was added into the test tube and 10 mL of boiling water was added and then cooled. The mixture was agitated vertically for 10 seconds. For 10 minutes formation of foam indicates saponins.

Test for triterpenoids

10 mg sample was mixed with 5 mL of ether solution and evaporated. Test solution was mixed with anhydrous acetate acid and strong H_2SO_4 (2:1). Formation of red-green color indicates triterpenoids.

Test for steroids

Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for cardiac glycosides (Keller-Killani test)

Five ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxy-sugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer

In-vitro Antioxidant assays

DPPH radical scavenging assay

The DPPH (1, 1-diphenyl-2- picrylhydrazyl) radical scavenging capacity of the various extract of *M. pumilus* Linn. was determined by the method of Alvi et al., (2016). Briefly the free radical scavenging activity based on the scavenging activity of the stable DPPH free radical. DPPH molecule determines with the occurrence of a purple color. DPPH solution (132 mM) was prepared in methanol in a dark reagent bottle. 100µl of the leaf, stem and fruit extracts from *M. pumilus* Linn. and ascorbic acid (Concentration ranging from 0.0 to 200 µg/ml) was added to 2 ml of DPPH solution and the reaction mixture was incubated for 15 minutes at 27ºC in a water bath and absorbance was measured at 517 nm. The reduced form of DPPH was generated, accompanied by the disappearance of the violet color. Ascorbic acid was used as a reference standard. Percent (%) scavenging of DPPH free radical was measured using the following equation.

$$\%\text{DPPH} = \frac{\Delta\text{Absorbance of control} - \Delta\text{absorbance of test sample}}{\Delta\text{Absorbance of control}} \times 100$$

Further, IC_{50} value represented the concentration of the extract that caused 50% inhibition of DPPH radicals and was calculated by interpolation of linear regression analysis using Origin Professional for Windows.

ABTS radical scavenging assay

Radical-scavenging activity of *M. pumilus* Linn. was determined according to the previously established protocol. ABTS radicals were pre generated by adding 5 mL of

a 4.9 mM potassium persulfate solution to 5 mL of a 14 mM ABTS solution and kept for 16 h in the dark. Different concentrations of extracts of *M. pumilus* Linn. (0.0–200 µg/mL) were added to the above activated pre-generated ABTS solution. This solution was suitably diluted with distilled water to yield an absorbance of 0.90 at 734 nm and then used for antioxidant assay. Ascorbic acid (0.0- 200 µg/mL) was used as a reference compound. 50 µL of extracts/standard was added to 950 µL of ABTS solution and vortexed for 10 s and after 6 min and then reduction in absorbance was recorded at 734 nm, using distilled water as a blank, on Eppendorf UV-visible spectrophotometer (Germany).

Acetylcholinesterase Inhibition Assay

A colorimetric assay for AChE enzyme was performed as described by Ellman et al. (1961) with some modifications (Alvi et al., 2016). In brief, 1 milliliter of reaction mixture was prepared by mixing, 100 µL of 10 mM DTNB (1 mM/reaction), 100 µL of 15 mM AChI (1.5 mM/reaction), 700 µL of 50 mM Tris HCl (pH 8.0) (35 mM/reaction), and 100 µL of varied concentrations of plant extract (0.0, 25, 50, 100, 200, and 400 µg/mL) into a 2 mL cuvette. Acetylcholine iodide (AChI; substrate) was used as substrate of AChE enzyme. The cuvette that consists of DTNB, buffer, and substrate was used as a ‘blank’, while another cuvette containing 25 µL of AChE enzyme solution 0.28 UmL−1 by substituting the equal volume of buffer was used for the analysis of product (nitro benzoate) formation. We used the standard drug tacrine for the comparative analysis. At a wavelength of 405 nm, this reaction was observed for 20 min after every 1 min interval in an Eppendorf BioSpectrometer (equipped with thermostatically controlled cell holder).

During the reaction, AChE hydrolyses the acetylcholine to produce thiocholine and acetate. The resulting thiocholine, in turn reduces the dithiobis-nitrobenzoic acid (DTNB), liberating nitro benzoate (yellow), which gets absorbed at 405 nm. The activity of AChE enzyme in the presence and absence of inhibitors was analyzed by measuring the product (nitro benzoate), which was formed after the reduction of DTNB by thiocholine. The amount of thiocholine produced in the reaction has nothing to do with the enzymatic active pocket once it is released from the same. Thus, the liberated

thiocholine cannot interfere with further enzymatic activity, and the color produced after the reaction between thiocholine and DTNB is thought to be the direct indication of the enzymatic activity. AChE activity is expressed in micromolar of AChI hydrolyzed per minute (U/min). The values that used for the calculation were the average of three replicates.

The percentage inhibition was calculated as described below:

$$\%\ Inhibition\ of\ AChE\ activity = \frac{\Delta\ \text{Abs. of control}\ -\Delta\ \text{Abs. of drug}}{\Delta\ \text{Abs. of control}} \times 100$$

Furthermore, the IC_{50} value represents the minimum concentration of the inhibitor that inhibits the 50% of AChE activity and was calculated using non-linear regression analysis interpolation through the Origin Professional for Windows.

Enzyme kinetic studies to determine the mode of inhibition of AChE

The varied concentrations of substrate, acetylcholine-iodide or AChI (*i.e*., 0.5, 1.0, and 2.0 mM), were used for the analysis of kinetic study of AChE activity and its inhibition by varied concentration (0.0, 200, and 400 µg/mL of reaction) of *M. pumilus* as at room temperature (25°C). Kinetic analysis of acetylcholine iodide hydrolyzed by AChE in the absence and presence of inhibitors was observed spectrophotometrically at a wavelength of 405 nm for a total of 20 min, and the absorbance values were recorded at 1 min intervals. Lineweaver Burk and Dixon plots were used to determine the kinetic parameters, such as Ki, Vmax, and Km values (Alvi et al., 2016).

Statistical analysis

For all assays, samples were analyzed in triplicates and the data were expressed as mean ± SD. The results were evaluated for stats using either GraphPad Prism version 4.02 for Windows (GraphPad Software, San Di-ego, USA) or Origin professional Software suit.

Results

Table 4: %Yield of phytochemicals in various extracts of *M. pumilus* Linn.

Extract	%Yield
n-Hexane	1.92
Dichloromethane	1.79
Ethyl acetate	1.61
MeOH	9.90
Aqueous	8.32

Table:5. Phytochemical constituents of *M. pumilus* Linn. fractions.

	n-Hexane	EtOAc	DCM	MeOH	Aqueous
Cardiac glycosides	-	++	+++	+	-
Steroids	-	-	-	-	-
Phenols	++	++	-	+++	-
Flavonoids	+++	++	-	++	-
Tannins	-	-	-	-	-
Saponins	-	-	-	++	-
Terpenoids	-	-	-	-	-
Quinones	-	-	-	-	-
Coumarins	+++	+++	-	++	+
Phlobatannins	-	-	-	-	-
Anthocynins	-	-	-	-	-

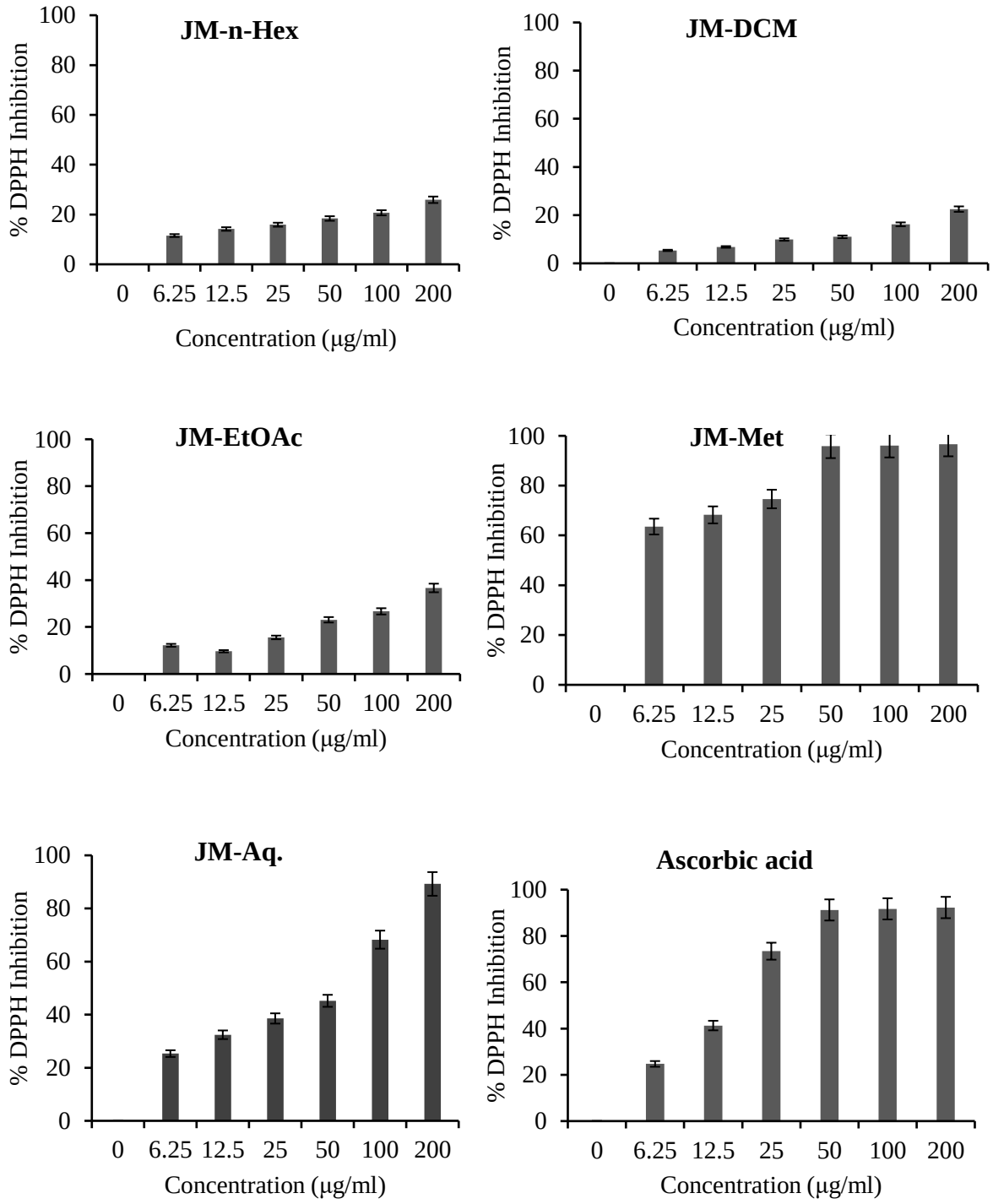


Figure 12: DPPH free radical scavenging activity of sequentially extracted *M. pumilus* Linn extracts. Data is representing the mean ± SEM. All the measurements were carried in triplicates.

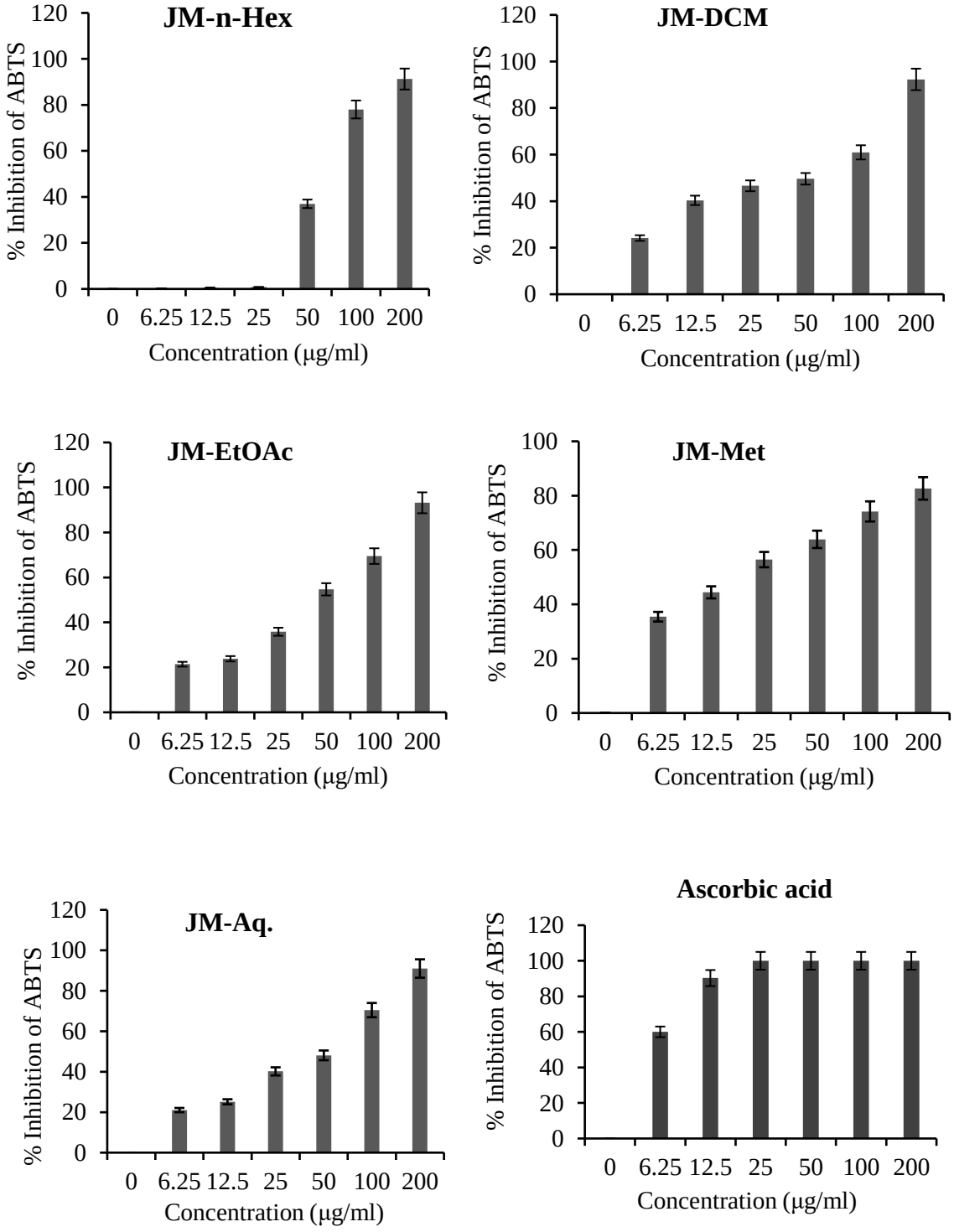


Figure 13: ABTS radical scavenging activity of sequentially extracted *M. pumilus* Linn extracts. Data is representing the mean ± SEM. All the measurements were carried in triplicates.

Table: 6. IC_{50} values of different extract of *M. pumilus* Linn. against DPPH and ABTS radicals.

Fractions	DPPH (IC_{50}: µg/ml)	ABTS (IC_{50}: µg/ml)
N- hexane	ND	66.40 ± 2.61
DCM	ND	53.12 ± 2.23
EtOAc	ND	43.91 ± 1.82
MeOH	4.47 ± 0.03	17.23 ± 0.81
Aqueous	59.821 ± 1.49	54.49 ± 2.52
Ascorbic acid	16.20 ± 0.08	4.80 ± 0.02

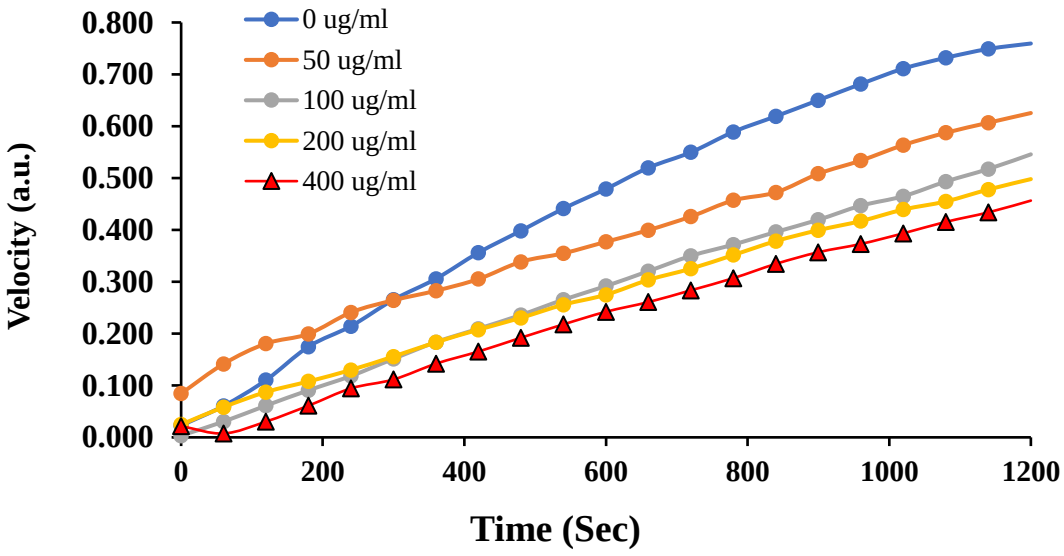


Figure 14: Time-scans demonstrating the progress of AChE-mediated hydrolysis of AchI either in presence or absence of *M. pumilus* Linn. Aqueous extract (JM-Aq).

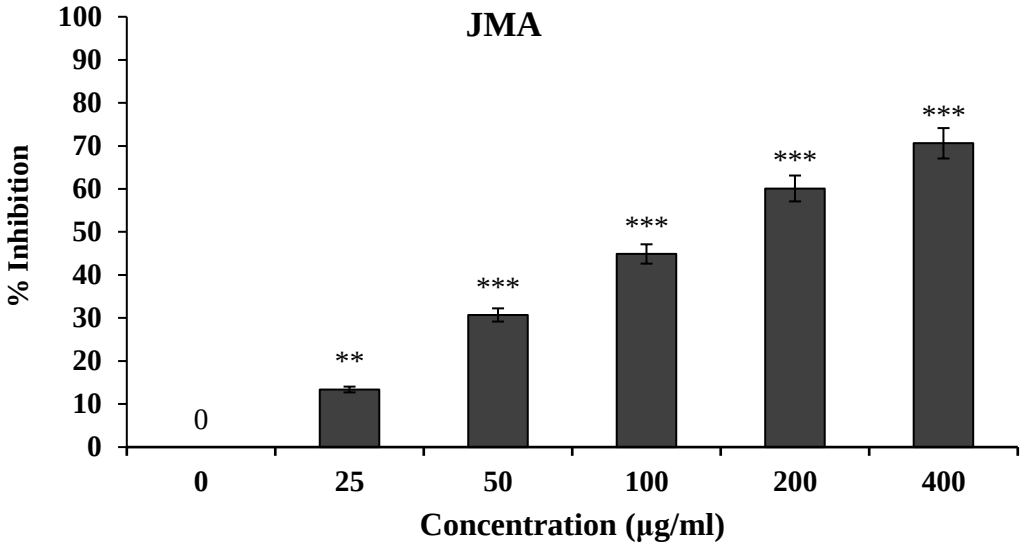


Figure 15: AChE inhibitory activity of various concentrations (0, 25, 50, 100, 200, and 400 µg/ml) of *M. pumilus* Linn. Aqueous extract (JM-Aq). Data is representing the mean ± SEM. All the measurements were carried in triplicates.

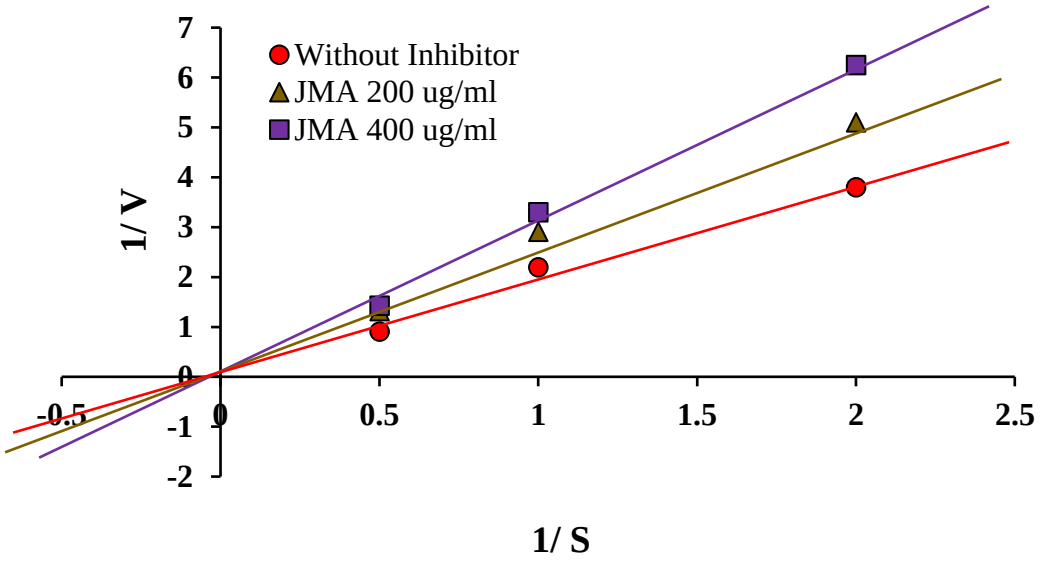


Figure 16: Lineweaver Burk Plot demonstrating the competitive mode of inhibition of AChE in absence or presence (200 and 400 µg/ml) of *M. pumilus* Linn. Aqueous extract (JMA).

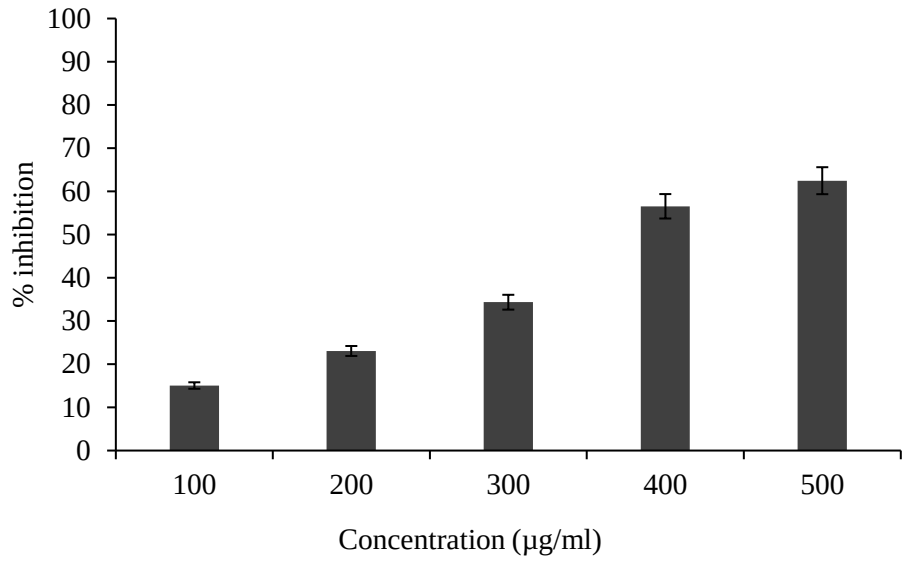


Figure 17: AChE inhibitory activity of various concentrations (0, 100, 200, 300, 400, and 500 µg/ml) of *M. pumilus* Linn. methanolic extract (JMM). Data is representing the mean ± SEM. All the measurements were carried in triplicates.

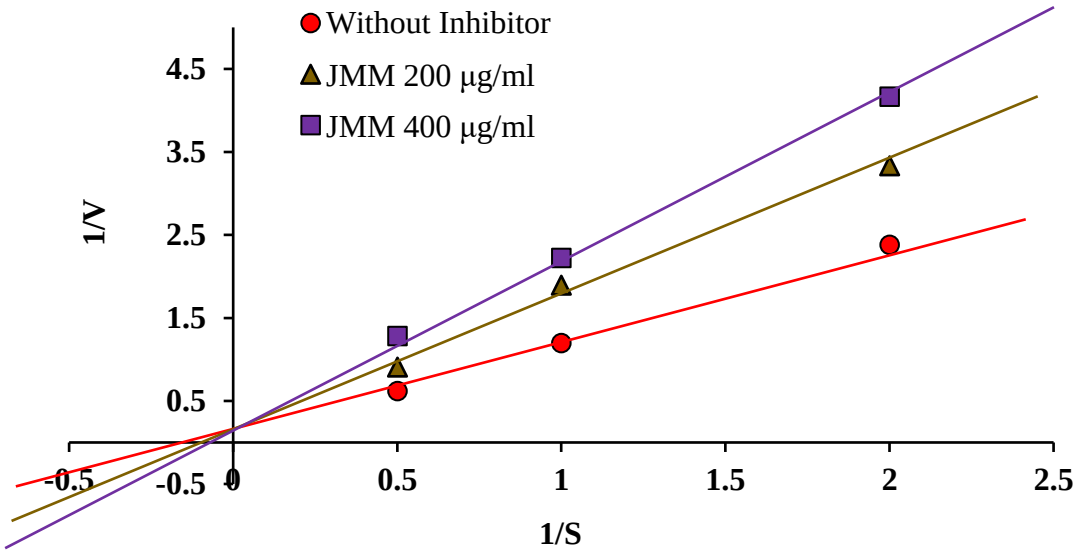


Figure 18: Lineweaver Burk Plot demonstrating the competitive mode of inhibition of AChE in absence or presence (200 and 400 µg/ml) of *M. pumilus* Linn. methanolic extract (JMM).

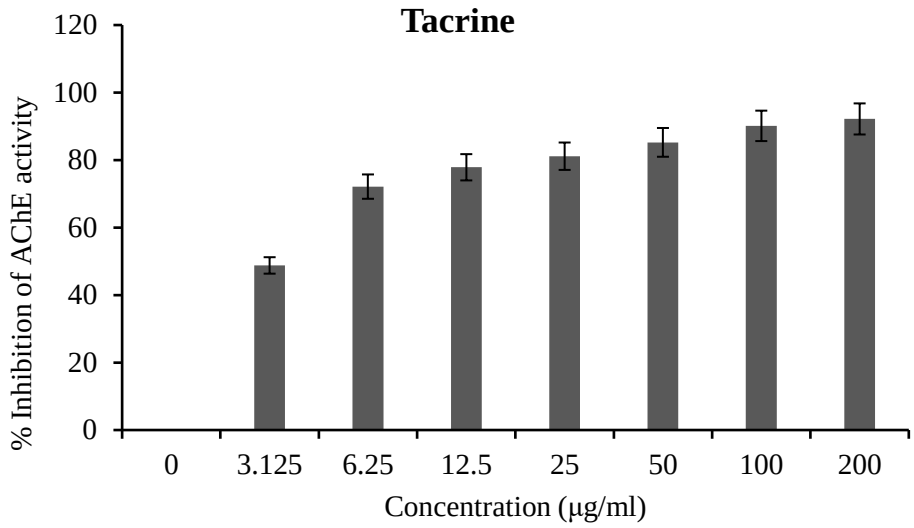


Figure 19: AChE inhibitory activity of reference standard tacrine (0, 25, 50, 100, and 200 µg/ml). Data is representing the mean ± SEM. All the measurements were carried in triplicates.

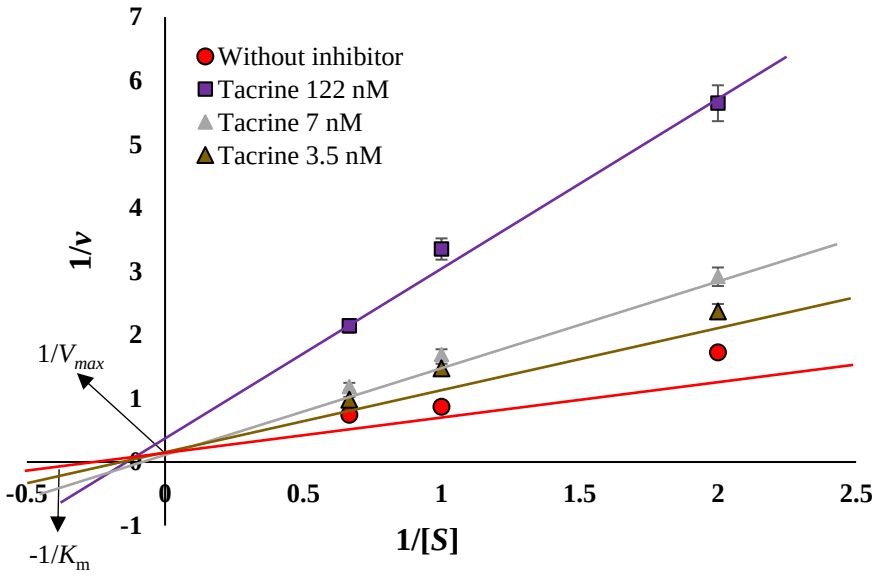


Figure 20: Lineweaver Burk Plot demonstrating the competitive mode of inhibition of AChE in absence or presence tacrine.

Discussion

Oxidative stress and inflammation are connected with numerous pathological conditions. Synthetic drugs accessible for curing these disorders cause numerous undesirable effects (Alvi et al., 2019; Alvi et al., 2016). A number of studies are being conducted worldwide to assess natural sources for the active or lead compounds with best safety profiles. Phytochemical analysis of *M. pumilus* Linn. extract was done in order to identify the presence of bioactive compounds such as flavonoids, phenols, tannins, and saponins. This is supported by Shahid et al., (2013) which revealed that *M. pumilus* Linn. was rich in bioactive compounds including phenols, sterols, carotenoids (*i.e*., lycopnen), anthocyanins, procyanins, flavonoids, tannins, carotenoids, alkaloids, and polyphenols which are good sources of antioxidant. The potential health benefits of *M. pumilus* Linn. have been partially attributed to their phenol contents, especially flavonoids that have received much attention from the literature over the past decade for its biological effects. The flavonoids and phenolic acids are known to possess antioxidant activities due to the presence of hydroxyl groups in their structures, and their contribution to the defense system against the oxidative damage due to endogenous free radicals is extremely important (Saggu et al., 2014; Alvi et al., 2016; Alvi et al., 2019).

Phenolic compounds or polyphenols and alkaloids are secondary plant metabolites that are ubiquitously present in plants and their products. Most of them have been proven to have high levels of antioxidant activities (Razali et al., 2008). Due to their redox properties, these compounds such as flavonoids, tannins, and alkaloids contribute to the overall antioxidant activities. The tabulated data of the present study was well supported with the previously reported findings (Shahid et al., 2013; Hashim et al., 2013). Medicinal plants are accepted as a vital source of new compounds having therapeutic potential. The research on folkloric usage of plants as pain relievers, anti-inflammatory and hepatoprotective agents should therefore be viewed as a fruitful research strategy for search of new analgesic, antioxidant, anti-inflammatory and hepatoprotective drugs.

DPPH radical scavenging activity is widely used to evaluate antioxidant activities in a relatively short time. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Alvi et al., 2016). The effects of the bioactive compounds from plants on DPPH radical scavenging are thought to be due to their hydrogen donating ability (Siddaraju & S. M. Dharmesh 2007). This method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule (Alvi et al., 2016). In the same context, we also analyzed the DPPH free radical scavenging activity of different extracts of *M. pumilus* Linn.

In this attempt, we reported that only methanolic and aqueous extracts of *M. pumilus* Linn. showed potent antioxidant activities with IC_{50} values of 4.47 ± 0.03 µg/ml and 59.821 ± 1.49 µg/ml, respectively. Such substantial DPPH radical scavenging activity of these two extracts of *M. pumilus* Linn. may be attributed to the presence of the bioactive secondary metabolites and the same could be corroborated with the presences of phytochemical profiling recorded in the current study. However, rest the extracts *i.e*., n-Hex, DCM, and EtOAc did not show any quenching effects against DPPH free radicals. Such findings of DPPH radicals quenching by methanolic and Aq. extracts of *M. pumilus* Linn. are in accordance with the previously published reports (Alvi et al., 2016). In contrast, standard drug *i.e.*, ascorbic acid also showed potent DPPH free radical scavenging activity with an IC_{50} 16.20 ± 0.08 µg/ml which was not as significant as reported in case of *M. pumilus* Linn. methanolic extract.

In addition to the DPPH free radical scavenging activity, the ABTS quenching ability of various extracts of *M. pumilus* Linn. was also assessed as ABTS is also another most widely used method for the determination of antioxidant potential of various plant extracts and their bioactive secondary metabolites. In this attempt, we reported that all the investigated extracts (i.e., n-Hex, DCM, EtOAc, Methanolic, and Aqueous) of *M. pumilus* Linn. exhibited strong ABTS radical scavenging activity with IC50 values of 66.40 ± 2.61 µg/ml, 53.12 ± 2.23 µg/ml, 43.91 ± 1.82 µg/ml, 17.23 ± 0.81 µg/ml, and 54.49 ± 2.52 µg/ml, respectively. Among these extracts, methanolic

extract of *M. pumilus* Linn. was the best quencher of ABTS radicals (17.23 ± 0.81 µg/ml). Such substantial ABTS quenching ability of *M. pumilus* Linn. extracts may be attributed to the existence of various secondary metabolites i.e., terpenoids, carotenoids, phenols, and flavonoids that are well-known for their antioxidant effects (Alvi et al., 2016; 2019; Ahmad et al., 2019; 2020). In contrast, reference standard ascorbic acid also showed potent ABTS radical scavenging activity with an IC50 of 4.80 ± 0.02 µg/ml.

It is worth mentioning that tacrine in 1993 received FDA approval as the first approved drug for Alzheimer's disease and acts as an AChE inhibitor; however, due to its various side effects, such as hepatotoxicity, it was discontinued in 2013 for disease treatment (Sharma K, 2019). Similarly, the other approved medications have several side effects, and due to the economic and safety of natural products, there is a continuous need to search for bioactive potential of the natural products or compounds derived from them. Prasad and Muralidhara found that co-administration of geraniol with curcumin has an inhibitory effect on AChE activity Prasad & Murlidhara 2014). Previous reports suggested that natural plant-based products, such as essential oils have shown neuroprotective effects through the inhibition of AChE (Alvi et al., 2019; Oboh et. al., 2014).

In the present study, the extracts of *M. pumilus* Linn. with highest DPPH and ABTS radical scavenging activity *i.e*., methanolic and Aqueous were selected for the assessment of their AChE inhibitory activity. In this attempt, we found that both methanolic and Aq. extracts of M. pumilus Linn. exhibited significant AChE inhibitory activity with IC_{50} values of 368.68 ± 5.02 µg/ml. and 133.26 ± 4.8.11 µg/ml, respectively. This beneficial AChE inhibitory activity of both these extracts of *M. pumilus* Linn. may be attributed to the presence of bioactive metabolites of this plant as we also reported the presence of various bioactive secondary metabolites in all the extracts. Similar AChE inhibitory activities of various plant extracts have previously been reported and were successfully correlated with their antioxidant potential (Alvi et al., 2019). However, tacrine (standard drug) also showed a significant AChE inhibitory

activity with an IC_{50} of 4.418 ± 0.15 µg/ml which is consistent with the previously published report (Iqbal et al., 2021).

Furthermore, the mode of inhibition of AChE activity by both these extracts as well as tacrine was also determined using enzyme kinetics studies and the findings were represented as the double reciprocal Lineweaver burk plot (1/S vs 1/V). Our enzyme kinetics investigations revealed that both methanolic and aqueous extracts of *M. pumilus* Linn. exhibited the competitive mode of inhibition for the inhibition of AChE activity. Which signified the potent therapeutic efficacy of the *M. pumilus* Linn. for the management of neurological disorders as well as oxidative stress related pathologies. On the other hand, tacrine also exhibited the competitive mode of inhibition for the inhibition of AChE activity an such findings of the current study are well justified by the previously published report (Iqbal et al., 2021). With regard to these earlier reports and our antioxidant and enzyme inhibition results, which suggest the bioactive potential of *M. pumilus* Linn., it was found that plants with AChE inhibitory and antioxidant activity may help in preventing or alleviating patients suffering from AD.

Conclusion

On the verge of side effects of synthetic acetylcholinesterase inhibitors, which are causing serious implications in Alzheimer patients, we need new concepts, new theories and new points of view rather than standard drugs because of their side effects. Our results for the first time reported the phytochemical screening, antioxidative studies and anti-acetylcholinesterase activity of *M. pumilus* Linn. We reported that only methanolic and aqueous extracts of *M. pumilus* Linn. showed potent antioxidant activities with IC_{50} values of 4.47 ± 0.03 µg/ml and 59.821 ± 1.49 µg/ml, respectively, when compared to the ascorbic acid which showed potent DPPH free radical scavenging activity with an IC_{50} 16.20 ± 0.08 µg/ml which was not as significant as reported in case of *M. pumilus* Linn. methanolic extract. Furthermore, we also reported that all the investigated extracts (*i.e*., n-Hex, DCM, EtOAc, Methanolic, and Aqueous) of *M. pumilus* Linn. exhibited strong ABTS radical scavenging activity with IC50 values of 66.40 ± 2.61 µg/ml, 53.12 ± 2.23 µg/ml, 43.91 ± 1.82 µg/ml, 17.23 ± 0.81 µg/ml, and 54.49 ± 2.52 µg/ml, respectively. Among these extracts, methanolic extract of *M. pumilus* Linn. was the best quencher of ABTS radicals (17.23 ± 0.81 µg/ml). In the present study, the methanolic and Aqueous extracts of *M. pumilus* Linn. with highest DPPH and ABTS radical scavenging activity exhibited significant AChE inhibitory activity with IC_{50} values of 368.68 ± 5.02 µg/ml. and 133.26 ± 4.8.11 µg/ml, respectively. Our enzyme kinetics investigations revealed that both methanolic and aqueous extracts of *M. pumilus* Linn. exhibited the competitive mode of inhibition for the inhibition of AChE activity. Which signified the potent therapeutic efficacy of the *M. pumilus* Linn. for the management of neurological disorders as well as oxidative stress related pathologies. Further, detailed in-vitro identification of bioactive metabolites, cell culture and in-vivo studies are required to make further decisions regarding the therapeutic application of this plant.

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